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Determination of Proliferative Characteristics of Growth Plate Chondrocytes by Labeling with Bromodeoxyuridine

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Summary. Postnatal bone growth occurs by the process of endochondral ossification in cartilaginous growth plates at the ends of long bones. The rate and extent of long bone growth is determined by a combination of chondrocytic proliferation, matrix production, and increase in chondrocytic height in the direction of growth during cellular enlargement. In this study, single pulse and/or repeated pulse labeling with the thymidine analog bromodeoxyuridine (BrdU) was used to study the role of cellular proliferation in controlling long bone growth. Variables studied included progression of the label over time following a pulse, and patterns and progression of the label over time following repeated pulse labeling for 24 and 48 hours. Examination was made of the proliferative characteristics of chondrocytes, the spatial pattern of cellular proliferation, and cell cycle kinetics. With respect to the spatial pattern of proliferative chondrocytes, results suggest that chondrocytes within a column are more synchronized with each other than are chondrocytes in different columns. This is consistent with the concept that each column represents a clonal expansion of a stem cell, which may proceed independently from adjacent columns. Despite this apparent heterogeneity, all chondrocytes in the proliferative zone complete at least one cell cycle in 24–28 hours. This estimate of the cell cycle time is significantly shorter than previous estimates of cell cycle times in similar growth plates. Our results also suggest that chondrocytes entering the cell cycle in the proximal part of the growth plate spend an average of 4 days in the proliferative cell zone, representing approximately four cellular divisions. After leaving the cell cycle, an additional 48 hours is required for the label to reach the terminal chondrocyte, which represents the time required to complete hypertrophy. These data are important when considering hypotheses concerning both the role of controls on proliferation in the determination of overall rate of long bone growth, as well as the interplay between proliferation and hypertrophy in regulating the overall amount of growth achieved by a given growth plate.

Key words: Chondrocyte – Growth plate – Bromodeoxyuridine – Proliferation.

Long bones grow postnatally by a process of endochondral ossification controlled by chondrocytes of the cartilaginous growth plate. Most long bones have both a proximal and a distal growth plate. Specific growth rates as well as the duration of growth differ in the two growth plates of one bone, and the amount of total growth contributed by each growth plate is characteristic of both the bone and the species. An additional complexity occurs in regions such as the antebrachium and crus. Proximal and distal growth plates of the radius/ulna and tibia/fibula achieve overall growth that is both coordinated between the two bones and that allows for overall equality of length to be achieved through differential contributions to growth (including both rate and duration) by the four growth plates. The control mechanisms that allow for coordinated bone growth throughout the body are incompletely understood, but probably involve all levels of organization from embryonic patterning, to systemic hormonal controls, and to local paracrine, autocrine, and biomechanical controls over growth plate activity [1–4].

In any given growth plate, chondrocytes occur in a characteristic spatial organization that is also a representation of the temporal progression of individual chondrocytic differentiation. A stem cell population of chondrocytes on the epiphyseal side of the growth plate contributes to a pool of proliferative cells that arrange themselves together in organized groups or columns. As a transition to terminal differentiation, each chondrocyte leaves the proliferative pool and, prior to its death at the chondroosseous junction, greatly increases in size and undergoes a characteristic shape change during the process classically described as hypertrophy [1, 2]. The rate and extent of growth for a given growth plate is determined by a combination of chondrocytic proliferation, matrix production with controlled degradation, and increase in chondrocytic height in the direction of growth during cellular enlargement [3–5].

Although chondrocytic proliferation clearly is *required* for long bone growth, it is not known to what extent systemic and local controls act at the level of the proliferative chondrocyte to *control* the rate and extent of long bone growth [6, 7]. There is the complexity of examining multiple growth plates growing at different rates in one animal at one point in time, versus examining any given growth plate growing at different rates throughout the period of postnatal growth [8]. The contribution of proliferation to growth of a single growth plate may vary significantly at different ages [3, 9]. Variables associated with proliferation that are necessary to understand overall control mechanisms throughout the period of growth, and which may change at different times postnatally, include characteristics of both the stem cell pool and of the proliferative cell pool such as size of the pool, whether the pool is heterogeneous or homogeneous, and cell cycle kinetics within the pool.

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The present study focuses specifically on the proliferative cell pool. In this study we develop a labeling method using single pulse and/or repeated pulse labeling with the thymidine analog bromodeoxyuridine (BrdU) to study characteristics of chondrocytes throughout the zone of chondrocytic proliferation, the spatial patterns of chondrocytic proliferative activity, and cell cycle kinetics [10]. It is our long-term goal to understand the relative contributions of chondrocytic proliferation, matrix metabolism, and cellular hypertrophy in controlling the overall rate and extent of postnatal long bone growth in normal animals, as well in diseases of postnatal bone growth. We believe this is the first time that BrdU labeling has been used to systematically study proliferative cell characteristics of chondrocytes within the growth plate, and to make *direct* measurements of cell cycle parameters of these cells.

Materials and Methods

Three-week-old Sprague Dawley rats, randomly chosen from both sexes, were used in this study. Because of circadian influences on proliferation, animals were kept in 12 hours light/12 hours dark [11–13]. For all experiments in which 24-hour intervals were analyzed, collections were made at lights on (6:00 a.m.).

For these experiments, the thymidine analog BrdU was used to label cells in the S phase of mitosis as an indicator of chondrocytes within the growth plate with proliferative potential. BrdU labeling, as opposed to tritiated thymidine incorporation, is a relatively new technique for the study of cell cycle kinetics. It has been used in a variety of both *in vivo* and *in vitro* analyses in multiple tissues and cellular types for the study of labeling indices associated with both development and with neoplasia [14–21]. An immunocytochemical technique for use on growth plates has previously been described, with specific modifications for use on Epon-embedded tissue [10, 22]. This technique has several well-established advantages compared with tritiated thymidine including relatively rapid processing times, lack of radioactivity, and the possibility of using a double labeling technique with either iododeoxyuridine or with tritiated thymidine [16, 23–26]. The present study is the first use of BrdU labeling for directly studying proliferative cell performance in the growth plate.

Bromodeoxyuridine was dissolved in sterile saline and injected intraperitoneally at (25–50 mg/kg) except for those experiments specifically designed to analyze different dosage levels of BrdU. Growth plate collections were made from the proximal tibia as previously described [8]. Each growth plate was trimmed to blocks measuring approximately 1 mm × 1 mm × 3 mm and included epiphyseal and metaphyseal bone on each end. Unless specifically indicated otherwise, for each time point of each analysis, growth plates were collected from two rats. Fixation was by immersion for 3 hours in 2% glutaraldehyde–2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.3, containing 0.7% ruthenium hexamine trichloride (RHT) [27]. After a buffer rinse, all growth plates were dehydrated in graded alcohols and cleared in propylene oxide. Infiltration was in 1:1 propylene oxide and Epon-araldite for 2 hours, followed by pure Epon-araldite for 3 days in a dessicator. Embedded blocks were polymerized at 60°C for 5 days.

Localization of incorporated BrdU was done with a monoclonal antibody on 1 μm-thick sections using a protocol modeled after that of Apte et al. [22]. Briefly, 1 μm-thick sections were placed on albumin-coated slides and etched with sodium ethoxide, which served both to allow penetration of the antibody, as well as to denature the DNA on the tissue section as required for this localization. Etched sections were rehydrated through graded alcohols and rinsed in phosphate-buffered saline. A commercially available anti-BrdU antibody (Beckton Dickinson, Mountain View, CA), supplied at a concentration of 25 μgm/ml, was diluted and run routinely at dilutions of both 1:50 and 1:100. Incubation was for 24 hours at 4°C. Specific localization was by using a biotin/avidin system with a peroxidase-labeled, secondary antibody from a commercially available kit (Zymed Laboratories, Inc., So. San Francisco, CA). Sections were mounted using a water-soluble mounting medium, and photographed with Technical Pan film, ASA 160.

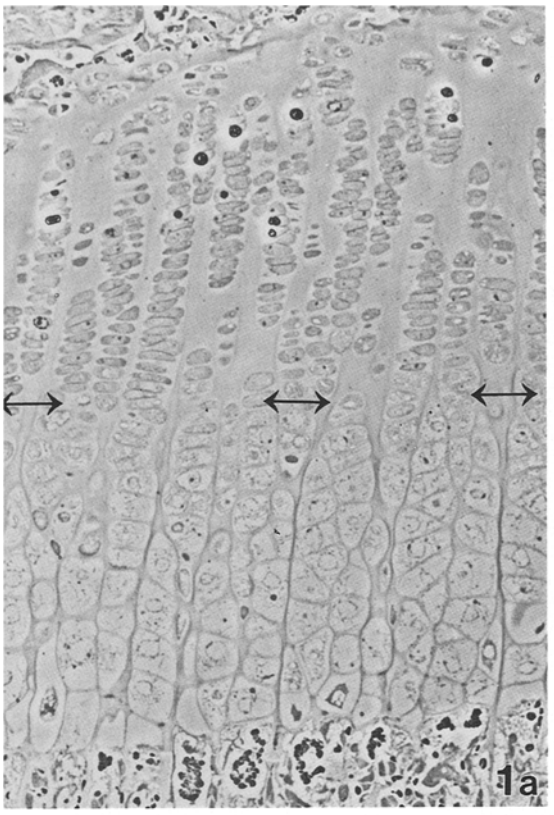
A series of experiments was performed to determine the optimal concentration of BrdU to use (1–100 mg/kg), as well as the optimal time of collection of tissue following BrdU labeling in order to satisfy the criteria of a "pulse" label. For the latter, time periods of 15, 30, 45, and 60 minutes postinjection were used.

Four kinds of experiments using single-pulse or repeated-pulse labeling with BrdU were performed. (1) One pulse of BrdU was given, and the progression of the label was followed over time. Intervals of 1, 2, 4, and 12 hours were examined. This yielded data on early time points after injection, and the maximal length of the S through M phases of the cycle. (2) Repeated pulse labels of BrdU were given every 4 hours, and the pattern of the progression of label was analyzed over a 48-hour period. This was an initial experiment to determine the time it would take to label all chondrocytes of the growth plate during at least one cycle. (3) Repeated pulse labels were given every 4 hours over a 24-hour period, and the progression of the label through the growth plate zones was followed every 24 hours for a total of 144 hours. This yielded data on progression of the label after *all* cells in the proliferative pool were labeled. (4) Comparison was made between one pulse label, followed every 24 hours for 144 total hours, and pulse injections given every 4 hours and followed for 144 hours. This experiment was important to determine if there was toxicity involved with repeated pulse labels of BrdU. The choice of 4-hour intervals for the repeated pulse was chosen based on data that the duration of the synthetic phase of the cell cycle (S phase, see below) was approximately 8 hours. Therefore, by pulsing every 4 hours, it was assured that no chondrocyte that reached the S phase could complete the S phase without exposure to a pulse. Experiments were terminated after 144 hours as preliminary data had indicated that by 120–144 hours after a pulse, essentially all label would have progressed throughout the growth plate.

A potential limitation of this technique is that of BrdU toxicity when given as repeated pulse labels over several days. A BrdU-mediated effect on phenotypic expression has been demonstrated for chondrocytes in culture [28, 29]. However, the data from the present study clearly indicate that chondrocytes from animals given BrdU proceeded through mitosis and also proceeded through their normal differentiative stages including hypertrophy. In addition, times of transit of the label through all zones was comparable in both single pulse-labeled and repeated pulse-labeled animals, indicating that it is unlikely that toxicity was a major problem.

For each time point for each experiment, two blocks were examined for each of two animals. On photographs at approximately 260×, the total area of the growth plate considered to represent cells of the proliferative cell zone was estimated as all chondrocytes from the first chondrocyte at the top of a column to the chondrocyte most distal to that column containing a label. The distal border of the proliferative cell zone corresponded to a clearly definable transition in cellular morphology in which initial shape changes leading to hypertrophy occur, and this transition could be identified easily even on sections that lacked a label (Fig. 1a).

From any one section, it is not possible to identify labeled cells unless the cellular profile contains a nuclear profile. This is demonstrated in Figure 2a–c where arrowheads point to three cellular profiles that are unlabeled, but which can be shown to contain label out of the plane of section. For the calculation of a labeling index (LI), for each section that was analyzed, labeled *nuclear profiles* were counted, as were all *cellular profiles*. A correction factor (to relate the probability of finding a nuclear profile within any given cellular profile) was determined by following 700 cells in serial sections and determining the number of cellular profiles containing nuclear profiles for each chondrocyte. The calculated correction factor was 0.395. Stereologically, this correction factor is equivalent to the ratio of the average caliper diameter of the nucleus to the average caliper diameter of the chondrocyte perpendicular to the plane of section ($\bar{\emptyset},\theta$) [30]. The LI for each experiment time period was then estimated as labeled nuclear profiles/total nuclear profiles in the proliferative cell zone. Labeling indices were plotted as a function of time. For specific experiments in which it was important to determine if 100% of chondrocytes of the proliferative pool were labeled, individual cells were followed on photographs of serial sections, magnified to approximately 500×.



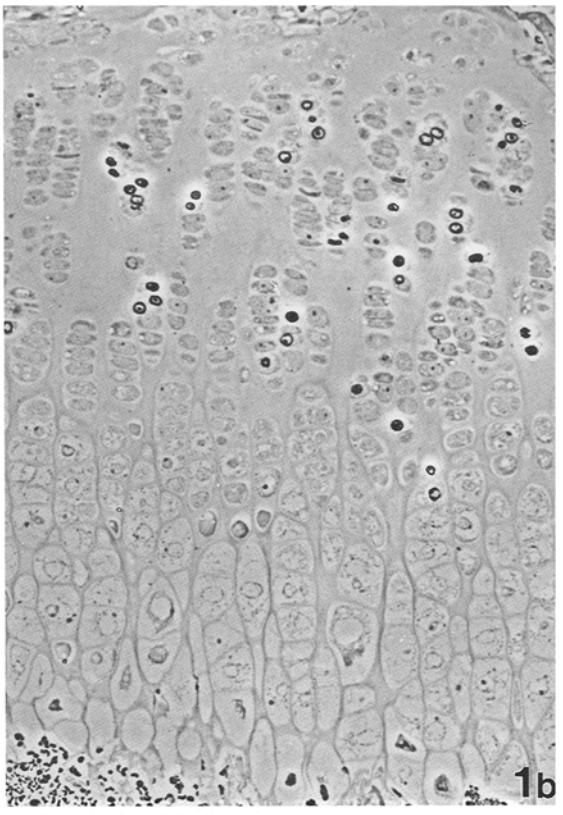
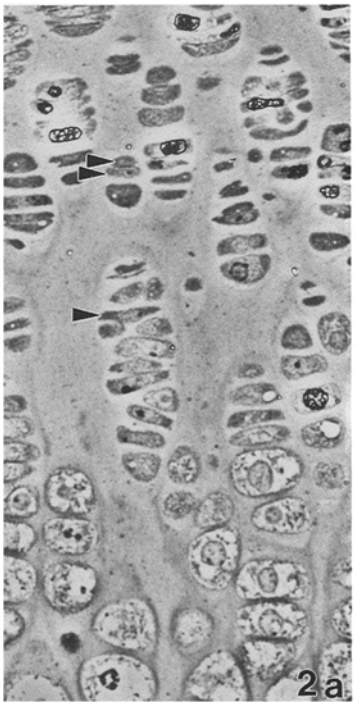
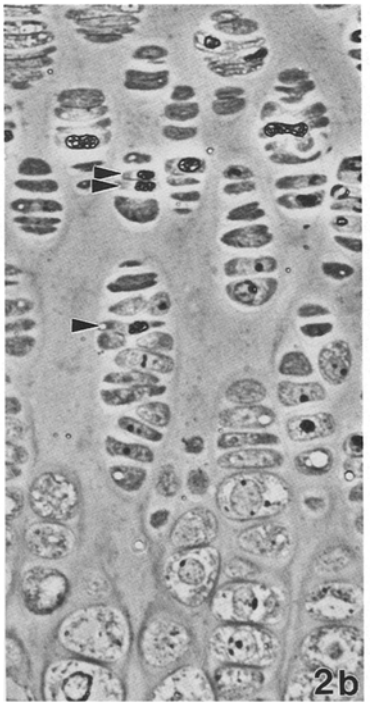


Fig. 1. The proliferative cell zone was considered to be all chondrocytes from the top of the columns to the transition to a shape change consistent with the onset of rapid cellular enlargement during hypertrophy (level of double-headed arrows, Fig. 1a). Given one pulse label, the LI is ≅ 15% (Fig. 1a). This doubles to approximately 30% by 8 hours after the label, and remains at approximately 30% through 24 hours (Fig. 1b). ×220.





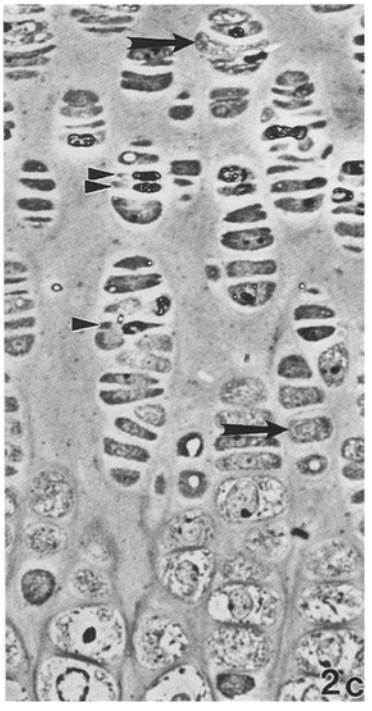


Fig. 2. From one section it is not possible to identify labeled cells unless the cellular profile contains a nuclear profile. Arrowheads point to three cellular profiles that are unlabeled in Figure 2a, but which contain label out of the plane of section (Fig. 2b,c). Two additional arrows in Figure 2c indicate chondrocytes that are unlabeled in this section, but which show a labeled nuclear profile in Figures 2a and b. ×440.

Results

There was no significant difference in either the percent of chondrocytes labeled in the proliferative zone nor in the pattern of labeling when collection times were 15, 30, 45, or 60 minutes following the BrdU injection. Collections were standardized at 30 minutes postlabeling for all experiments. Labeling dosages of 0.1, 4.0, 10.0, 20.0, 50.0, and 100 mg/kg were tested. Consistent labeling was achieved for all dosages of 20 mg/kg or more. To standardize, dosages of 50 mg/kg were used for single pulse label experiments, and 25 mg/kg for each dosage during repeated pulse label experiments.

Single Pulse Labeling Experiments: Progression of the Label and Labeling Patterns

Given one pulse label, the LI is approximately 15% (Fig. 1a). This doubles to approximately 30% by eight hours after the label, and remains at approximately 30% through 24 hours (Fig. 1b). This indicates that the duration of the S phase through the M phase (mitosis of the cell cycle) is approximately 8 hours. Mitotic figures are visible (Fig. 3a,b) indicating that labeled cells are capable of division. By 12 hours after the pulse, pairs of cells with equal distribution of the label are seen (Fig. 4a,b), and these presumably represent two daughters of chondrocytes originally labeled during the S phase. Forty-eight hours are required before the first label reaches the terminal chondrocyte (Fig. 5a–c). Label could be demonstrated both in hydrated terminal chondrocytes (Fig. 5a,b), as well as terminal cells in the condensed form that represents chondrocytes dying before capillary penetration at the chondroosseous junction (Fig. 5c) [31]. This indicates that the time interval required to go through hypertrophy (from when a proliferative chondrocyte leaves the cell cycle to when it dies at the chondroosseous junction) is approximately 48 hours. By 96–120 hours, all label has left the proliferative cell zone. This suggests that once a chondrocyte enters the cell cycle it remains cycling for about 96 hours. This time course of progression of a single pulse label through the proliferative zone is given in Graph 1.

The distance between the labeled cell positioned most proximally in the column and the cell positioned most distally following one pulse defines the extent of the proliferative cell zone. Using this definition, the distal extent of the zone is just proximal to the point of rapid transition to cellular hypertrophy, as can be seen in Figure 6a. After one pulse, there usually was a higher frequency of label in cells positioned proximally in the column compared with the cells closer to the transition to cellular hypertrophy (Fig. 6b). Over short periods of from 2 to 4 hours, chondrocytes in different columns were not highly synchronized with each other, but chondrocytes within a column were. Often a regular pattern of cellular division within one column was visible (Fig. 7a,b). On any given section, adjacent columns varied in both the extent and the pattern of the label, with significantly more total label in some columns than in others (Fig. 8a). This difference of labeling between columns could be observed even in the distal hypertrophic cell zone (Fig. 8b).

Repeated Pulse Labeling Experiments: Progression of the Label

Given a repeated pulse every 4 hours, all chondrocytes in the proliferative pool demonstrated label within 24–28 hours, resulting in a labeling index of essentially 100% over this time period (Fig. 9a,b). This could be demonstrated by calculations using the nuclear profile correction factor of 0.395 to relate nuclear profiles to cellular profiles (LI ≅ 95% after 28 hours, Graph 2). LI of 100% was confirmed by direct serial section analysis (Fig. 9a,b).

If chondrocytes were given repeated pulse labels every 4 hours for 24 hours and then labeling was stopped, progression of the label could be followed throughout all zones. As with the single pulse, 48 hours were required before label could be found in the terminal hypertrophic chondrocyte. By 72 hours, essentially all chondrocytes of the hypertrophic zone were labeled. By 96 hours, the LI of proliferative zone dropped to 30%. All label had left the proliferative zone by 120 hours. These results are presented in Graph 3. Even 96–120 hours after a 24-hour repeated pulse label, sporadic labeling could be seen in the proximal part of the proliferative cell zone, indicating renewed clonal expansion of a stem cell (Fig. 10a,b).

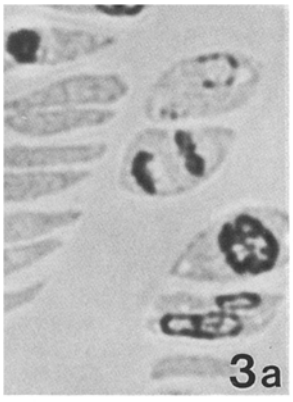
Discussion

A primary difficulty encountered when trying to analyze the role of proliferative cell activity in the control of the rate and extent of mammalian long bone growth is the complex interplay of several variables. A given growth plate has (1) a characteristic rate of growth at any point in time, and this is different for each growth plate of the body; (2) a characteristic pattern of growth rate over time, from birth to growth plate closure [3, 4, 6, 7, 9]; and (3) a circadian rhythm to many specific metabolic functions such as DNA and matrix synthesis [11–13, 32–35]. The design of the present study was to control for time and growth plate location by studying one growth plate at one point in time, and to examine cell cycle parameters directly through the BrdU labeling technique. Using this approach, several results emerge that suggest that chondrocytic mitotic activity within the growth plate is more complex than previously thought. It is important to discuss these results by comparison with previous studies on growth plate cartilage designed to study proliferative characteristics of growth plate chondrocytes.

BrdU Labeling for the Study of Cell Cycle Kinetics in Growth Plate Chondrocytes

There are potential limitations to the quantitative calculation of the LI based on BrdU incorporation used in the present study. First, there is the problem of defining the proliferative zone consistently on all sections. Using one criterion, the proliferative zone can be defined as extending from the most proximally labeled cell on a section to the most distally labeled cell in all the columns on that section. This allows an unequivocal demarcation for the population on any given photograph because it is based on direct observation of uptake of label, such as in Figure 6a. However, there is the problem that, due to the probabilistic nature of cellular labeling, on many sections no chondrocytes in the distal part of the columns on a given photographic section label, such as in Figure 6b. On this kind of section, if the criterion of distance between the proximally and the most distally labeled cell is used, there is an underestimation of the size of the proliferative zone, and a corresponding overestimation of the labeling index.

To obviate this problem, a second criterion to use is the morphological one associated with the zone of the initial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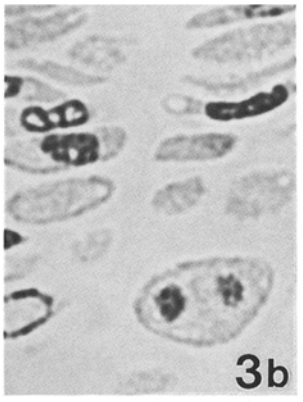
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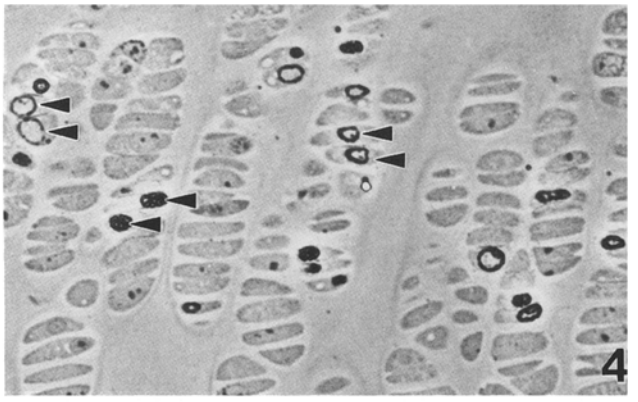
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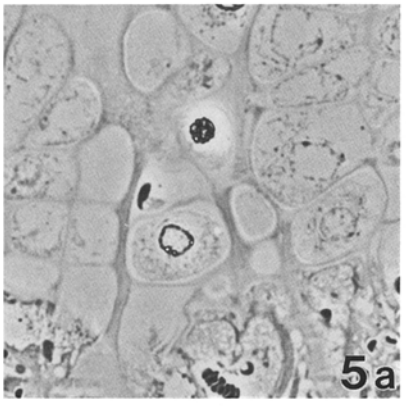
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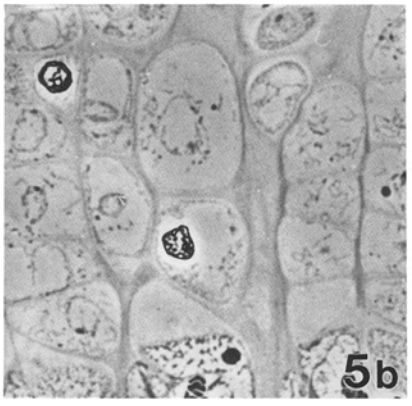
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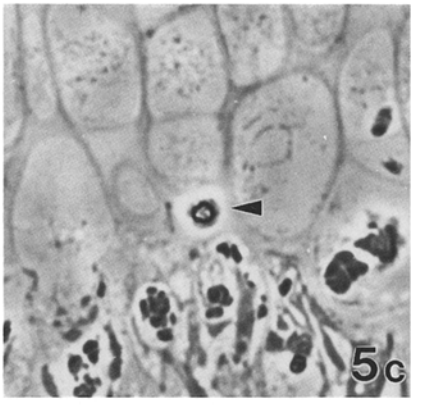
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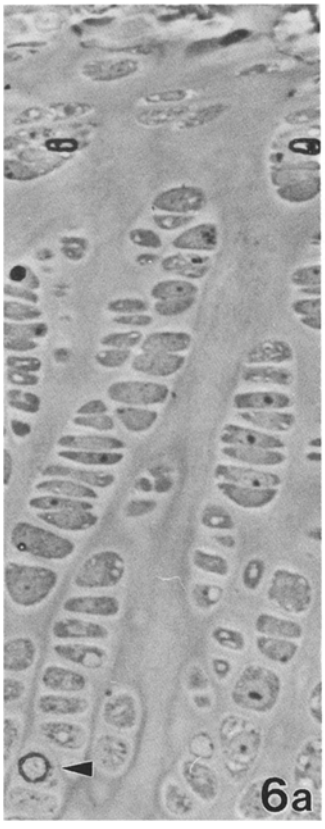
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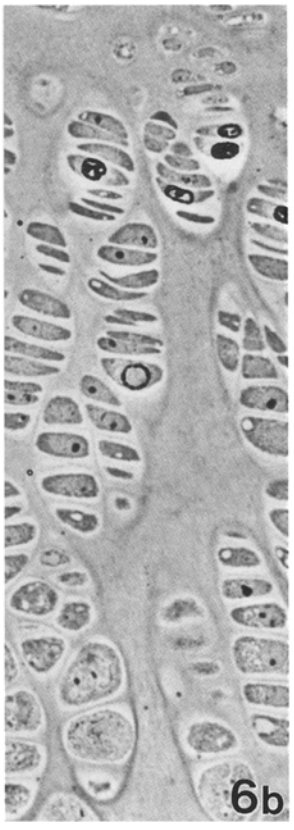
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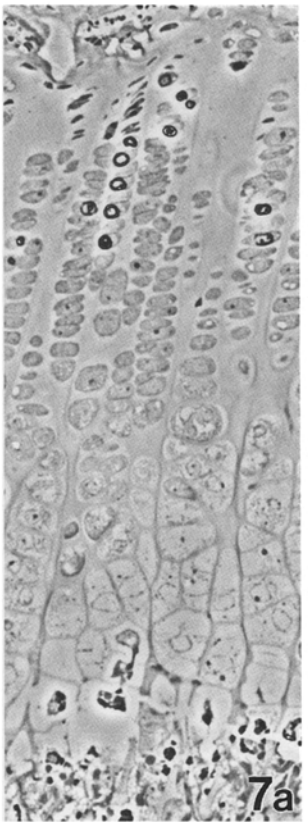
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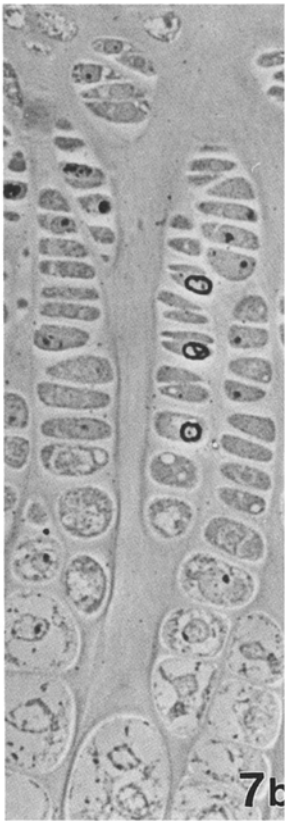
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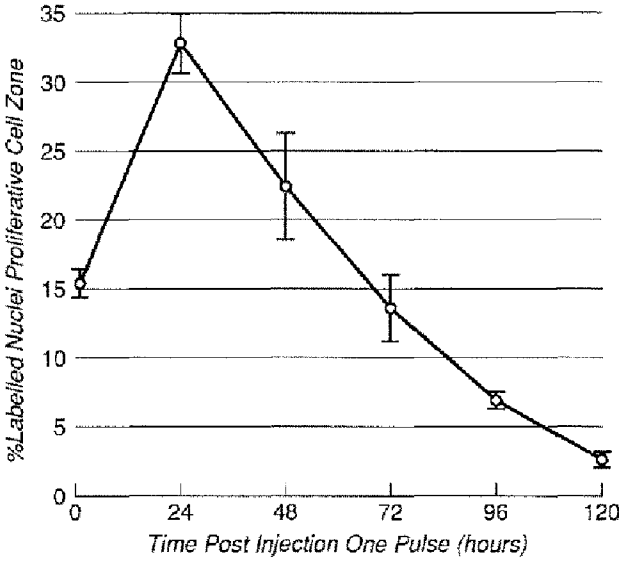


Graph 1. Time course of progression of a single label through the proliferative cell zone. For each time point approximately 1400 proliferative cell profiles were examined from each of two rats. 0 = mean ± standard error of the mean.

shape change leading to cellular hypertrophy. The morphology consistent with this transition is straightforward to identify on all photographs, and corresponds within two to three chondrocytes to the most distal extent of potential labeling after a pulse label. In the present study, this morphological criterion was used to define the distal boundary to the proliferative zone, and that way consistent demarcations could be made on all sections. It can be argued that using this criterion overextends the distal boundary of the true proliferative cell zone, and can therefore lead to an underestimation of the labeling index, causing a consistent, systematic error in the results. What is needed is a reliable second, independent marker to unambiguously define the proliferative cell zone. Although other proliferative cell antigens such as PCNA and Ki67 have been described [15, 36–39], neither of these has reactivity under the experimental conditions of this study using Epon-embedded sections and fixation with ruthenium hexamine trichloride.

A second limitation in the calculation of LI is the use of a correction factor to relate total cellular profiles to total nuclear profiles. Though we believe that this correction factor is valid for the overall proliferative population of the proximal tibia of the young rat, it may not be accurate for each population of cells in different experimental analyses. For example, there is a calculated LI of slightly greater than 100% for some experiments, which indicates an underestimation of total nuclear profiles. One alternative would be to stain unlabeled nuclear profiles so that they are clearly visible in the photographs and could be counted directly. To date, we have not been able to find a consistent nuclear stain for tissue fixed in the presence of ruthenium hexamine trichloride and Epon-embedded sections that allows good identification of all nuclear profiles. The alternative of serial section analysis is not practical given the large number of animals required for this kind of study. For instances where the calculated LI slightly exceeded 100%, analysis by serial section confirmed that all cells of the proliferative pool had taken up the label.

A final limitation to the use of a correction factor together with single pulse labels is that it is known that there are significant diurnal rhythms to the timing of DNA synthesis (and hence peak S phase activity) within the growth plate [11–13, 32, 34, 35]. Maxima of DNA synthesis peak around the transition to dark and in the early dark period, and LIs may be more than double compared with those achieved in the transition to light. In the present study, pulse labeling was standardized at 1 hour after lights on, and so calculated LIs should represent minimal values. In experiments in which repeated pulse labeling was achieved, analyses done over multiples of 24-hour intervals should avoid the interpretative constraints caused by diurnal variations.

Previous Studies on Chondrocytic Performance in the Growth Plate, Including Cell Cycle Analysis

Studies by Kember [40–42] beginning over two decades ago and using tritiated thymidine to label chondrocytes in the S phase of mitosis emphasized the significance of chondrocytic proliferation in the control of long bone growth. A major conclusion from these studies was that in mammalian growth plates, in general, labeling indices within the proliferative cell pool do not vary significantly in growth plates growing at different rates in a given animal at a given age. However, a major variable is the size of the proliferative cell population. This observation—that rapidly growing growth plates have larger numbers of and longer columns of proliferative cells than do more slowly growing plates—has been a major concept to be considered in the understanding of overall growth plate kinetics and the regulation of long bone growth [4]. In a more recent study of multiple growth plates in the rabbit, it was concluded that controls on proliferation rate were not independent of controls on the size of the proliferative cell zone. In that study, both LI, as well as the

Fig. 3. Mitotic figures are visible in Figure 3a,b, indicating that labeled cells are capable of division. ×880.

Fig. 4. By 12 hours after a single pulse, pairs of apparent daughter cells with equal distribution of the label are seen (arrowheads). ×440.

Fig. 5. Forty-eight hours are required before label from a pulse can be found in the terminal hypertrophic chondrocyte. Figure 5a and b demonstrate labeled nuclei in terminal chondrocytes at the chondroosseous junction; Figure 5c demonstrates label in a condensed (dying) terminal chondrocyte (arrowhead). ×220.

Fig. 6. The distance between labeled chondrocytes following one pulse can unequivocally define the extent of the proliferative cell zone. The arrowhead in Figure 6a indicates the most distally positioned chondrocyte in these columns, and this coincides with the region of transition to enlargement during hypertrophy. Notice the higher frequency of label in chondrocytes positioned proximally in the column, compared with chondrocytes positioned more distally in the proliferative cell zone (Fig. 6b). In Figure 6b, the most distally labeled chondrocyte is several cells proximal to the transition to cellular enlargement. ×440.

Fig. 7. Over short labeling periods of 2–4 hours, chondrocytes in different columns were not highly synchronized with each other, but chondrocytes within a column were (Fig. 7a, 7b). Also note the very regular pattern of chondrocytic replication in some individual columns. This is consistent with the concept that each column may represent a clonal expansion of a stem cell, which may proceed somewhat independently from adjacent columns. 7a, ×220; 7b, ×440.

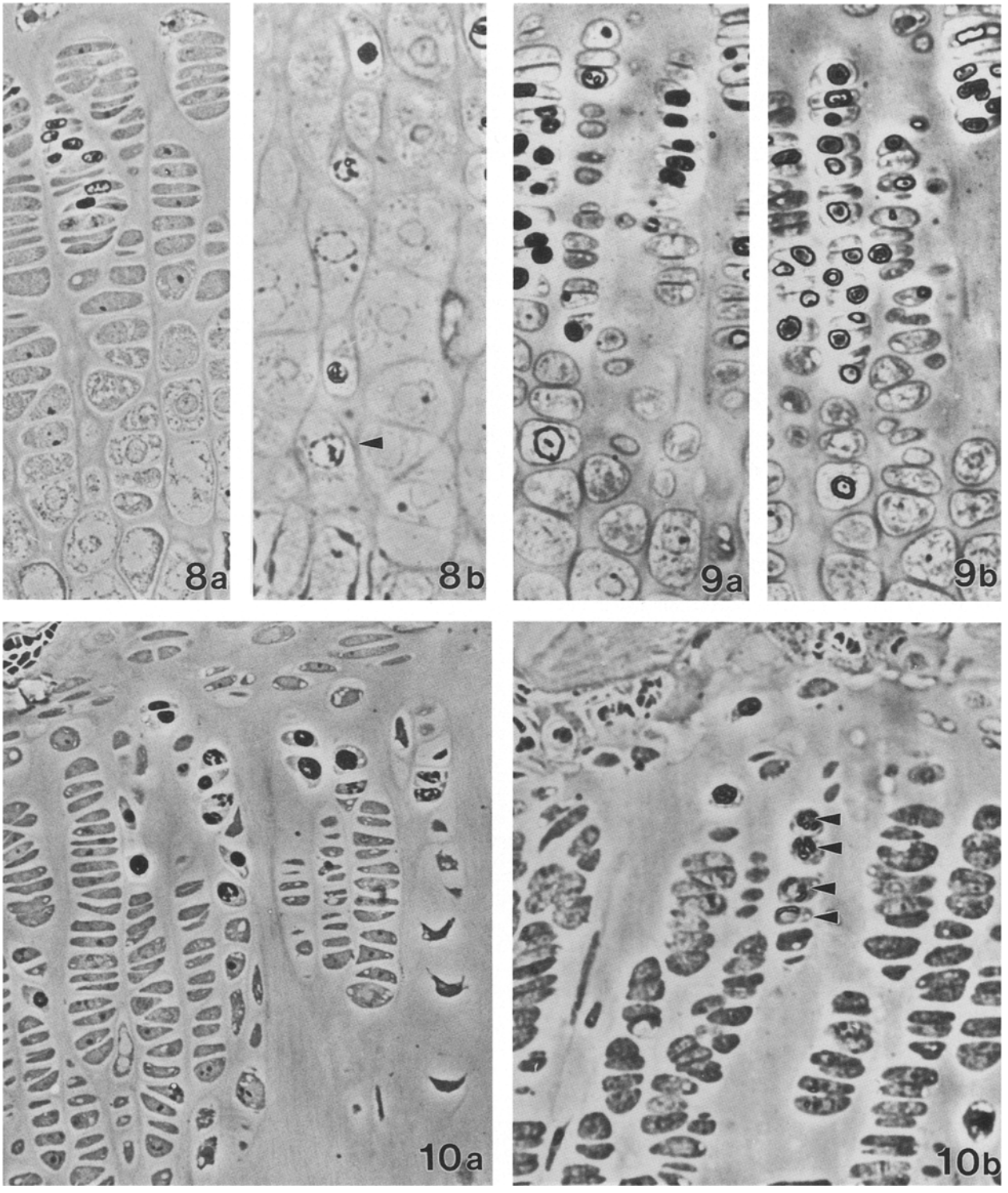
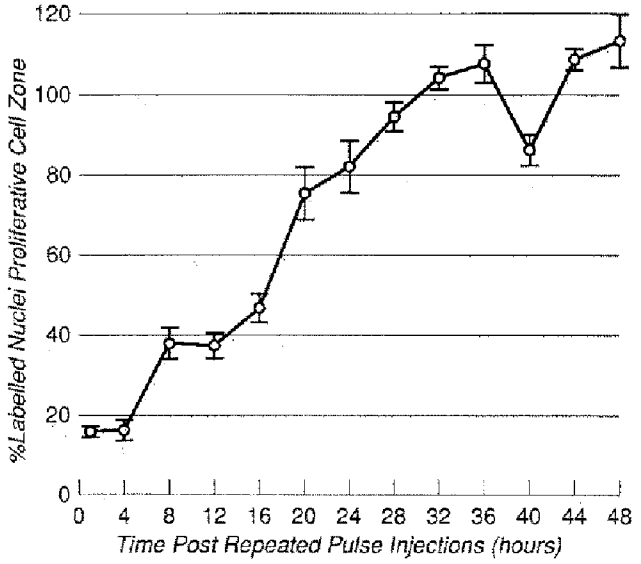


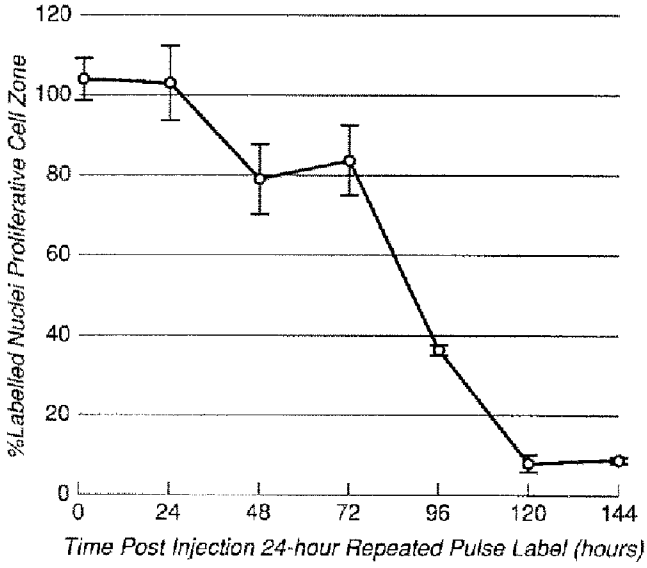
Fig. 8. Adjacent columns varied in the extent and pattern of the label. There was significantly more total label in some columns than in others (Fig. 8a). Notice that this difference of labeling between columns could be observed even in the distal hypertrophic cell zone (Fig. 8b). This micrograph shows one column of chondrocytes in which the majority of cells are labeled, including the terminal cell (arrowhead). Chondrocytes in adjacent columns lack label. ×440.

Fig. 9. After repeated pulse labeling for 24 hours, it could be demonstrated by serial sections that approximately 100% of chondrocytes of the proliferative cell zone contain the label. Notice that not all cellular profiles contain a nuclear profile, and so the extent of labeling can only be shown by following chondrocytes in serial section.

Fig. 10. Even 96–120 hours following 24 hours of repeated pulse labeling, sporadic labeling could be seen in the proximal part of the proliferative cell zone (arrowheads), indicating renewed clonal expansion of a stem cell. ×440.



Graph 2. Time course of cumulative labeling of the proliferative zone following repeated pulse labels. For each time point approximately 1400 proliferative cell profiles were examined from each of two rats. 0 = mean ± standard error of the mean.



Graph 3. Progression of repeated pulse labels (given for 24 hours) through the proliferative cell zone. For each time point approximately 1400 proliferative cell profiles were examined from each of two rats. 0 = mean ± standard error of the mean.

size of the pool, varied in growth plates growing at different rates, with higher labeling indices linked to longer proliferative zones [42].

Although controls acting at the level of the proliferative chondrocyte are *required* for the characteristic pattern of growth to be maintained, differential rates of growth at one time or over time may be *regulated* primarily through shape and volume changes during cellular hypertrophy. Recent studies have shown a high correlation and positive linear relationship between the final volume of hypertrophic chondrocytes and rate of longitudinal bone growth. In the proximal tibia of 3-week old rats, over 90% of growth is accounted for by the cumulative incremental changes in vertical cellular height in the direction of growth as a cell goes through the transition from a proliferative cell to a hypertrophic cell [8]. Proliferation in this model appears to function only to replace chondrocytes that die at the chondroosseous junction. This also is consistent with the observation that the actual rate of turnover of cells at the chondroosseous junction does not necessarily vary in growth plates growing at different rates, suggesting that actual growth is independent of cycles of cellular birth and death [3, 43].

Although in the 3-week-old rat cellular hypertrophy is the primary determinant of the actual amounts of growth achieved, the relative contributions of proliferation and hypertrophy to overall growth *may vary significantly at different ages and in different species*. Walker and Kember [9] found no reduction in either the duration of the S phase of mitosis nor the length of the proliferative zone for the proximal tibia of the rat from 21 to 91 days. Because growth rate of the bone decreases in this period, they concluded that slowing of growth over time was caused by a decrease in the cellular proliferation rate. However, they made no estimates of actual cell cycle times. Hunziker and Schenk [3] examined the question of control of growth during endochondral ossification by analyzing multiple parameters in the proximal tibial growth plate of the rat at three different ages (21, 35, and 80 days), representing very different rates of growth (276, 330, and 85 μm/day, respectively). The increase in vertical height during hypertrophy was shown to be a major determinant of the overall rate of growth at all three ages. In attempting to analyze the overall contributions of proliferation, net matrix synthesis, and chondrocytic height increase, these authors used *indirect methods* to estimate cell cycle times that varied at these three ages (81, 54, and 54 hours, respectively). Their conclusion was based on the assumption that cell cycle times do vary through time in a single growth plate and were highest at 3 weeks of age. However, cell cycle times were not measured directly in their study, but were calculated based on cellular production rates that would be required to achieve known rates of growth as measured by calcein labeling, and based on known rates of chondrocytic elimination per day at the chondro-osseous junction.

Relationship of Data from the Present Study to Previous Studies

A significant finding in the present study from the observations of labeling patterns of single pulse labeling is that, although all cells of the proliferative pool are capable of division, at any one time chondrocytes positioned more distally in a column appear to have a lower probability of labeling than those positioned proximally. If this is true, then one interpretation is that the duration of the cell cycle time lengthens between each division and that distal cells cycle significantly slower than proximal cells. Slowing of cell cycle times over time is a feature of cells in culture [44]. Data from the present study indicate that in the growth plate the entire life span of a cycling cell is only 4–5 days, a very short period compared with chondrocytes in culture. An alternative explanation is that individual chondrocytes may permanently leave the proliferative pool at different times so that a smaller fraction of chondrocytes positioned distally is capable of division compared with proximally. The authors favor the latter interpretation based primarily on the evidence that, for cells given one pulse and completing the first cycle, there

is a maximum LI of 30%; the pool is subsequently diluted after 48 hours as the cells move into the zone of hypertrophy.

Data from the present study indicate that the timing of DNA synthesis is more synchronous for chondrocytes within a column than for chondrocytes in different columns (even at the same level of the column). This is consistent with the concept that each column represents a clonal expansion of a stem cell, which may proceed independently from adjacent columns [5, 45, 46]. This was apparent not only from the different number of chondrocytes labeled in different columns after one pulse, but also because of the characteristic regular patterning of proliferative activity within a given column, as shown in Fig. 7a,b. Variables associated with cell cycle kinetics of the stem cell pool were not examined in the present study, but clearly these are major variables associated with long-term controls of growth, especially as they relate to slowing of growth and growth plate closure [5, 46]. The data from the present study, however, provide evidence of the clonal expansion of stem cells as, following one pulse, when essentially all label had disappeared from the growth plate, scattered areas of labeled columns were evident in the proximal part of the proliferative cell zone, representing division of a stem cell initiated approximately 5 days after the initial pulse (Fig. 10a,b).

Despite the possibility of variability of activity between columns and within a column at one instant in time, the present study demonstrates that, given a repeated pulse label, all chondrocytes in the proliferative pool label within 24–28 hours. It is not possible to discriminate between a cell that has taken up the label in the S phase and daughter cells that have label as a consequence of division of a previously labeled cell that has just gone through the M phase. However, there is the additional observation that after one pulse label, the zero time labeling index of approximately 15% doubles by 8 hours to approximately 30%. This would indicate that the duration of the S phase through M is approximately 8 hours. This agrees well with previous data suggesting S phase durations of 6–8 hours for these growth plates [33, 35]. Therefore, data from the present study suggest that cell cycle times are approximately 24 hours for chondrocytes in this growth plate. There is also the possibility that the duration of the cell cycle itself might have a diurnal variation so that, although the LI (measuring cells in the S phase) might vary at different points over a 24-hour period, the total number of cells completing a cycle might be relatively constant over a 24-hour period. Data from the present study are consistent with this interpretation.

Estimates of cell cycle times made in the present study are significantly shorter than the indirect estimate of 54 hours made previously in rats of the same age [3]. Similarly, using the method of counting pulse-labeled mitoses, in Wistar rats, Walker and Kember [35] calculated a 55 ± 40-hour cell cycle time of the proximal tibia in the 6-week-old rat. They concluded that there was a long mean cell cycle time and a wide distribution of cell cycle values around the mean. Both Walker and Kember [35] and Hunziker and Schenk [3] used rats of the Wistar strain, compared with rats of the Sprague-Dawley strain used in the current study. However, although not impossible to rule out, it is our opinion that strain differences would not account for cell cycle differences of greater than twofold, as the two strains have comparable rates of growth. Walker and Kember also used continuous injections of tritiated thymidine (0.25 μg/g body weight) at intervals of approximately 25 hours for up to 5 days. In one experiment, continuous labeling yielded approximately 50% of all cells labeling by 24 hours, and 80% by 48 hours. LIs never exceeded 80%. The analysis of LI was done on 5 μm sections, so there is a possibility of underestimation of the LI due to self-absorption of beta radiation in the thickness of the section. In another experiment, labeling reached 60% in 24 hours for chondrocytes positioned about halfway down the proliferative cell column, and these same cells reached a 100% LI after 3 days of continuous labeling [33]. Based on averages for labeled cell counts at different positions within the column, these investigators concluded that most all of the chondrocytes had completed their second division before 3 days, with a mean value of about 2 days. In another series of experiments designed to study circadian influence on chondrocytic proliferation, Simmons et al. [11] estimated a cell cycle time of approximately 36 hours based on calculations made using pulses of tritiated thymidine in multiples of 6-hour intervals.

In the present study, our calculation of LI utilized a correction factor that was based on the probability of seeing nuclear profiles in 1-μm thick sections. Furthermore, serial section analysis was an unequivocal and direct approach for analyzing all cells of the proliferative cell zone. Therefore, the data from the present study provide evidence that cell cycle times in these animals was significantly shorter than previously estimated, and that all cells in the proliferative pool were actively dividing.

The present study makes a contribution to understanding growth plate kinetics by making direct measurements of the total time a chondrocyte spends from entering the proliferative cell pool until death at the chondroosseous junction. Experiments with both single-pulse as well as repeated-pulse labeling gave consistent data that maximally 96–120 hours is spent in the proliferative pool. Based on cell cycle times estimated in the present study, this would represent an average of 4–5 cellular divisions. An additional 48–72 hours is required for chondrocytes to hypertrophy. Hunziker and Schenk [3] calculated that the duration of the hypertrophic phase of chondrocytic activity is 48 hours in the 35-day-old rat, based on the number of cells in the hypertrophic zone and the number of chondrocytes lost in a 24-hour period. This value was found to be fairly constant, and independent of animal age or growth rate. The interpretation, both from the Hunziker and Schenk study as well as from the present study, is that volume increase with regulated shape change is a primary controlling mechanism for achieving growth. These data are important when considering hypotheses concerning the regulation of proliferative cell activity, as well as in determining the overall significance of the balance between proliferation and hypertrophy in determining the rate and extent of overall long bone growth.

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