Journal of Physiology (1989), **414**, *pp.* 55–71
With 4 text-figures
Printed in Great Britain

PHYSIOLOGICAL MECHANISMS ADOPTED BY CHONDROCYTES IN REGULATING LONGITUDINAL BONE GROWTH IN RATS

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(*Received 5 April 1988*)

SUMMARY

1. Chondrocyte activities within growth plate cartilage are the principal determinants of longitudinal bone growth, and it was the aim of this investigation to assess how these cell activities are modulated under various growth rate conditions. Using proximal tibial growth plates from rats of different ages, growth rate was determined by fluorochrome labelling and incident light fluorescence microscopy. Various cellular parameters contributing to longitudinal bone growth were quantified by light microscopic stereology. The size of the proliferating cell population ('growth fraction') was estimated by autoradiography (using [^{3}H]thymidine labelling).

2. A comparison between data for suckling (21-day-old) and fast-growing (35-day-old) rats revealed that growth acceleration is achieved almost exclusively by cell-shape modelling, namely by an increase in final cell height and a decrease in lateral diameter, whereas final cell volume and surface area are slightly reduced. Cell proliferation rate in the longitudinal direction and net matrix production per cell remain unchanged. The physiological increase in linear growth rate thus appears to be based principally upon a controlled structural modulation of the chondrocyte phenotype. On the other hand, a physiological reduction in growth rate (i.e. growth deceleration) effected during the transition from pre-puberty (35-day-old rats) to maturity (80-day-old rats) is achieved by simultaneous decreases in several chondrocyte parameters, including cell height (i.e. phenotype modulation), cell volume and proliferation rate (in the longitudinal direction). However, chondrocytes continue to produce matrix at a level comparable to that attained during the period characterized by high growth rates (i.e. at 21 and 35 days). Cartilage matrix thus appears to play a subordinate role in *regulating* longitudinal bone growth rate. The duration of the hypertrophic cell activity (i.e. phenotype modulation) phase remains constant (at approximately 2 days) under the various growth rate conditions.

3. The findings presented in this study indicate that measurement of bulk parameters such as [^{35}S]sulphate incorporation into matrix components, [^{3}H]thymidine uptake by cells and growth plate height are of limited value as estimators of longitudinal bone growth, since changes in the parameters that these measurements quantify bear little relationship to changes in *linear* growth rate, and may be useful only as indicators of *total* growth plate activity.

INTRODUCTION

Longitudinal bone growth is regulated principally by genetic and endocrine factors influencing the activity of chondrocytes within growth plate cartilage, a disc-like structure interposed between the epi- and metaphyses of long bones (Hansson, 1967; Kember, 1978; Stockwell, 1979). Within their lifespan, chondrocytes perform their various activities in a defined sequence which occurs in synchrony with that of their lateral neighbours (Hunziker, Schenk & Cruz-Orive, 1987). Consequently, the growth plate cell population is structured into zones of similar morphology lying perpendicular to the longitudinal bone axis (Fig. 1). In the vertical direction too, chondrocytes are highly organized into columns, which are generally believed to represent the functional units for longitudinal bone growth (Hansson, 1967; Kember & Walker, 1971; Kember, 1978, 1983; Stockwell, 1979). The means by which chondrocytes modulate longitudinal bone growth is unknown, but changes in cell proliferation rate, height, volume and matrix production have been generally implicated (Hansson, 1967; Kember, 1983; Hunziker *et al.* 1987). The mechanisms adopted could be envisaged to follow one of three hypothetical courses. Growth, for example, could be regulated exclusively by changes in cell proliferation rate with other activities (such as matrix production, cell height and volume increases) operating continually at a fixed, maximal performance level. Alternatively, cell synthetic activities (including changes in height and volume) may be the site for control, under conditions of fixed proliferation rates. It is also conceivable that all cell activities are subject to some degree of regulation.

It is the aim of the current investigation to determine how the various chondrocyte activities are modulated under various growth rate conditions, and hence to elucidate the physiological mechanism(s) adopted by these cells for regulating longitudinal bone growth. In relation to the presented findings, the usefulness of measuring bulk parameters, such as total growth plate cell proliferation and matrix production, commonly used as indicators of longitudinal bone growth, is assessed.

METHODS

Animals

Eighteen female Wistar rats, six from each of three age groups (see below), were labelled with calceine (Fluka, Switzerland; 15 mg/kg body weight) for growth rate determination, 5 days prior to killing by an overdose of ether. On the day they were killed, rats were aged 21, 35 and 80 days, with body weights of approximately 30, 120 and 300 g respectively.

Preparation and processing of tissue blocks for stereology

Details of these procedures have been published previously (Hunziker, Herrmann & Schenk, 1982; Cruz-Orive & Hunziker, 1986; Hunziker *et al.* 1987), and are here summarized only. In order to determine growth plate reference volumes, frontal and sagittal diameters of exposed proximal epiphyseal plates were measured using a mechanical sliding calliper (Tesa, Switzerland; accuracy, $\sim 10\ \mu m$). Vertical tissue slices, cut in the sagittal plane through the proximal tibiae of rats, were transferred immediately to 2% (v/v) glutaraldehyde solution (in 0·05 M-sodium cacodylate buffer; pH 7·4) supplemented with 0·7% (w/v) ruthenium hexamine trichloride (RHT, Johnson Matthey, UK), and maintained at ambient temperature (Hunziker *et al.* 1982). Tissue slices, continually immersed, were further dissected with the aid of a stereomicroscope into small prismatic tissue blocks, with the long sides parallel to the longitudinal tibial axis. Blocks were maintained for 2–3 h in this primary fixation medium prior to washing (in isotonic, 0·1 M-sodium

cacodylate buffer). They were then post-fixed in 1% (w/v) osmium tetroxide solution (in 0·1 M-sodium cacodylate buffer; pH 7·4; 330 mosM) containing 0·7% (w/v) RHT, dehydrated in a graded series of ethanol and subsequently embedded in Epon 812. Dehydration was initiated in 70% ethanol in order to avoid proteoglycan solubilization (which occurs at lower ethanol concentrations; Hunziker *et al.* 1982). Moreover, the swelling problem previously reported to occur at ethanol concentrations below 70% (Boyde, Bailey, Jones & Tamarin, 1977) is thus obviated. Using a Reichert OMU3 microtome, sections were cut parallel to the tibial (i.e. vertical) axis, the section angle being oriented randomly relative to the horizontal plane for each block (Cruz-Orive & Hunziker, 1986). Thick sections were stained with Toluidine Blue O (Hunziker *et al.* 1982). For electron microscopic illustrations, thin sections were cut and stained with uranyl acetate and lead citrate.

Tissue slices (one per tibia) used for determination of longitudinal growth rate by incident light fluorescence microscopy were processed according to a different protocol (cf. section 'Determination of longitudinal growth rates').

Section thickness, determined using a Leitz–Michelson-interference phase-contrast microscope for incident light, was found to be 1·05 μm (c.v. = 3·9%, $n = 21$). Calibration of magnification in the light microscope was achieved using a Wild stage micrometer.

Quantification of chondrocyte performance

Determination of longitudinal growth rates. Calcein is a fluorescent label which binds specifically to actively mineralizing matrix at the time of application (i.e. 5 days prior to killing, see section 'Animals'). Measurements of the distance between the label front (determined using a Zeiss incident light fluorescence microscope equipped with a micrometric eyepiece; Hansson, 1967; Hunziker *et al.* 1987) and the lower end of the growth plate (where mineralization begins) thus provides an estimate of longitudinal bone growth during this period of time. Division of this value by 5 (days) gives an index of daily growth rate. This calculation is based upon the reasonable assumption that growth rate is constant during the 5 day period over which it was measured (Walker & Kember, 1972*a*; Nevo & Laron, 1979; Smith, Laurence & Rudland, 1981). Small diurnal variations in mitotic activity have previously been shown to be negligible (Walker & Kember, 1972*b*; Kember, 1978, 1983; Simmons, Arsenis, Whitson, Kahn, Boskey & Gollub, 1983).

Tissue slices used for determination of longitudinal growth rate were prepared as follows. A tissue slice was chosen by systematic random sampling from each of the two proximal epiphyseal plates of every animal (i.e. eighteen), and fixed in 40% (v/v) ethanol for 3 days at ambient temperature. Tissue slices were subsequently dehydrated and embedded in methyl-methacrylate, which was polymerized at +30 °C. Ten micrometre thick vertical sections were cut on a rotatory microtome (Jung, FRG) for microscopic examination and measurement.

Morphologic definitions. Two tissue blocks from each leg were chosen by a systematic random sampling procedure, cut, and photographed in the light microscope. Zone boundaries (defined below) were marked on paper prints at a final magnification of ×130. The layer of chondrocytes adjacent to the epiphyseal bone, consisting of cells occurring singly or in groups of two, and lying relatively unoriented with respect to the longitudinal axis, is defined as the resting zone (Fig. 1). Cells within this region have previously been shown to have stem cell function, and rarely undergo division (Kember, 1960, 1978, 1983). The adjacent proliferating zone is characterized by cells highly organized into columns oriented parallel to the longitudinal bone axis. They are of uniform height, relatively flat, and frequently undergo cell division by mitosis (determined in radiolabelling studies; Kember & Walker, 1971; Kember, 1972, 1978; Walker & Kember, 1972*a*). Following the proliferating phase, chondrocytes cease to divide, and begin to increase their height and volume. These hypertrophic activities are expressed within the final zone, which extends to the metaphyseal front of ingrowing blood vessels, and is arithmetically subdivided into an upper and lower half (Hunziker *et al.* 1987).

Stereologic estimators. A general problem associated with stereological data obtained from processed tissue sections is that shrinkage and hence volume changes occurring during preparative procedures may render such information invalid for native tissue. In a comparative study such as that presented here, it is essential to establish that (possible) shrinkage effects (i.e. volume changes) are constant both between the different rat age groups and between the various zones of individual growth plates. A useful artifact by which such changes may be monitored is the intracellular vacuole. We estimated the total cellular volume occupied by such vacuoles using quantitative

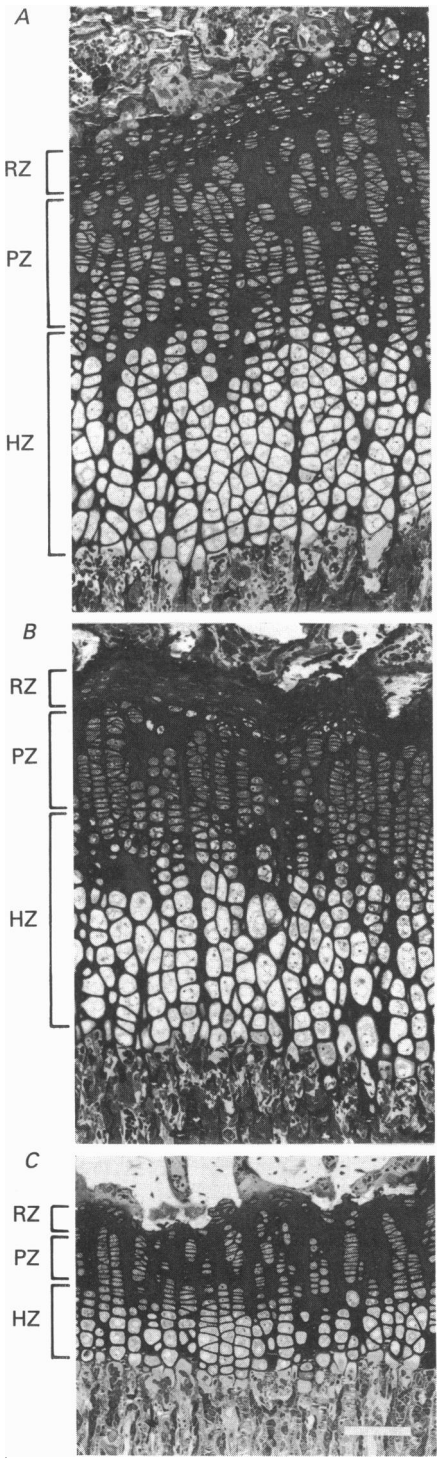

Fig. 1. Vertical section through the proximal tibial growth plate of a 21-day-old (*A*), 35-day-old (*B*) and 80-day-old (*C*) rat. Abbreviations: resting zone (RZ); proliferating zone (PZ); hypertrophic zone (HZ). Light micrographs of thick (1 μm) sections, stained with Toluidine Blue O and shown at *identical* magnifications ($\times$ 110). Bar = 100 μm.

electron microscopic morphology, and found that changes in this parameter, both between zones of an individual growth plate and between age groups, were constant (data not shown).

The heights of the growth plate (τ(gpl)) and individual zones were estimated by point counting from the height of the proliferating zone, which was determined by autoradiography (see below).

The number of chondrocytes (N(c)) within the proliferating and hypertrophic zones (V(str)) was estimated using the disector method (Sterio, 1984; Cruz-Orive & Hunziker, 1986), which utilizes a 'three-dimensional probe', i.e. two thick (1 μm) sections (S1 and S2) a known distance apart. The number of cells present in the reference section (S1), but no longer apparent in the other (S2), divided by the volume of the disector (i.e. the space between the two sections), gives an estimate of the number of cells per unit of disector volume. The total surface area (S(c)) occupied by chondrocytes in each zone (V(str)) was estimated by intersection counting, using a system of cycloid test arcs (Baddeley, Gundersen & Cruz-Orive, 1986; Cruz-Orive & Hunziker, 1986). When this procedure is adopted, an unbiased estimation of cellular surfaces can be obtained from cell profiles, provided that the cycloid's vertical axis is aligned with the vertical axis of the section (and that the verticality of the section is defined; in the present case this corresponds to the bone axis). Under these conditions, the cell surface area is proportional to the number of intersections between cell profile boundaries and cycloid arcs. The total volume (V(c)) occupied by chondrocytes in each zone (V(str)) was determined by point counting.

Cell shape parameters, such as mean equatorial (i.e. projected horizontal) diameter ($X(90°)$) and mean vertical height ($X(0°)$) cannot be determined by direct measurement on histological sections. The observed dimensions of each cell profile are characteristic of this profile *only*, and not of the chondrocyte as a whole, since profile diameter and height will vary as a function of the direction and depth of sectioning through each cell.

An estimate of the mean projected horizontal diameter of a chondrocyte ($X(90°)$) may be made using the expression: $X(90°) = N_A(90°)/N_V$ (cf. Cruz-Orive & Hunziker, 1986), where $N_A(90°)$ is the number of cell profiles present within a unit zone area of a vertical section through the growth plate, and N_V is the number of chondrocytes within a unit zone volume.

Approximate estimates of mean vertical cell height ($X(0°)$) were obtained using an oblate spheroid model for proliferating chondrocytes and a super-egg model for lower hypertrophic chondrocytes (Cruz-Orive & Hunziker, 1986). Since no unbiased model-free stereological estimation procedures are available for determining vertical cell height parameters, the methods applied are necessarily assumption-dependent (cf. Cruz-Orive & Hunziker, 1986).

Stereologic estimators for the hypertrophic phase of chondrocyte activity were determined exclusively for the terminal stages, i.e. the lower half of the hypertrophic zone. Since hypertrophy is a continuous process, the estimation of mean cell height ($X(0°)$) in the lower hypertrophic zone represents an average value between the terminal three to five chondrocytes.

The number of cells (n(c)) in a vertical cell column (within a given zone) was obtained using the relationship: $n(c) \simeq \bar{\tau}(str)/X(0°)$ (Cruz-Orive & Hunziker, 1986). This equation is not, however, applicable to the upper half of the hypertrophic zone, since due to the tremendous variation in cell shape found here, no appropriate model for estimating cell height can be found. An estimation of mean cell height within this zone was, however, made using an alternative approach. The profile heights of all upper hypertrophic chondrocytes cut centrally (through the nucleus) were recorded on histograms ($\sim$ 2000 profile heights per six animals), and the mean cell height (assumed to be similar to profile height at *this* point) determined. Due to the bias involved in the calculation of cell number per column within the upper hypertrophic zone, this parameter was also determined directly by counting the number of cells within complete column profiles in the section plane. Although a minor sampling bias is involved in this latter approach, both procedures yielded similar results.

The number of test points and intersections counted per compartment for each animal was in the range 100–200 (Cruz-Orive & Hunziker, 1986). The significance of differences between means of estimators for different age groups was calculated using Wilcoxon's signed rank test.

Some of the stereologic estimators determined for 35-day-old rats have appeared in a previous publication (Hunziker *et al.* 1987), describing the basic histophysiology of growth plate cartilage.

Cell kinetics

A parameter of basic importance in cell kinetic analyses is the 'growth fraction', which consists of the cell pool exhibiting a high mitotic activity (Mendelsohn, 1960; Barrett, 1966; Steel, 1967). In growth plate cartilage, this is defined as the proliferating zone (Kember & Walker, 1971; Kember, 1972, 1983).

The morphological extension of the proliferating zone was determined by [^{3}H]thymidine autoradiography. Two animals per age group were given intraperitoneal injections of [^{3}H]thymidine (Amersham International; 1 μCi/g body weight) 1 h prior to killing. Tissue blocks were fixed in buffered glutaraldehyde (2% v/v), dehydrated in a graded series of ethanol and embedded in Epon 812. One micrometre thick sections were exposed to Kodak-NT B 2-emulsion for 6 weeks prior to development. Labelled cell nuclei yielded the basis for delineating the proliferating zone (Fig. 1) from the adjacent resting and hypertrophic zones (consisting of unlabelled cell nuclei).

The 'columnar growth fraction' is defined as the total number of proliferating chondrocytes within a vertical cell column. Stem cells of the resting zone are not included in the columnar growth fraction, since their cycle times have been shown to be longer than the time period over which growth rate measurements were made (Kember, 1960; Walker & Kember, 1972*b*). And indeed, over short (1 h) periods of measurement of ^{3}H incorporation, labelling of resting cell nuclei was virtually zero. Moreover, these cells do not participate structurally in column formation.

The mean cell cycle time of proliferating chondrocytes is calculated by dividing the number of proliferating chondrocytes (within a column) by the number of chondrocytes eliminated per hour (Kember, 1960, 1983). This calculation gives an overall value for cell cycle time and cannot account for possible individual variations or the existence of differences between subpopulations of cells.

Calculations relating to cell kinetics are based upon the following assumptions, justification for which has previously been given (see references under 1 and 2 below).

(1) The vertical cell columns within cartilage growth plates act as functional units for the promotion of longitudinal growth (Kember & Walker, 1971; Kember, 1983).

(2) Within each vertical cell column, the rate of cell elimination is equivalent to the rate of cell production over short periods of time (such as 5 days, i.e. the time period over which longitudinal bone growth was measured; Walker & Kember, 1972*a*; Smith *et al.* 1981; Kember, 1983), and it is thus a measure of cell turnover. Mean cell turnover per column per day can thus be calculated from the daily cell elimination rate, which is equal to daily linear growth rate divided by final mean cell height (Steel, 1967; Walker & Kember, 1972*b*; Kember, 1983). The basic estimator of daily columnar cell turnover is thus obtained by a mathematical process combining growth rate measurements with stereologic parameters. Another example of this is the estimation of the mean duration of the hypertrophic phase of a chondrocyte. This is obtained by dividing the number of hypertrophic cells (within a column) by the number of cells eliminated per hour (i.e. the daily cell elimination rate per column divided by 24 h). Cell elimination rate is defined here as the number of terminal chondrocytes eliminated, in the sense of being excluded, from growth plate cartilage. Although the fate of these cells is unknown (Hunziker, Herrmann, Schenk, Müller & Moor, 1984), they certainly do not persist as chondrocytes.

RESULTS

Rat age groups were selected to coincide with the suckling (21 days), pre-pubertal growth spurt (35 days) and mature (80 days) phases in the lifespan of this animal (Kember, 1973, 1983). Each stage is characterized by specific numerical values with respect to body weight, growth rate and epiphyseal plate height (cf. Table 1 and Fig. 1), and it is apparent from the presented data that changes in growth rate do not necessarily correspond to changes in growth plate height as previously assumed (Greenspan, Li, Simpson & Evans, 1949; Phillips & Weiss, 1982).

The stereologic estimators describing the dimensions ((vertical) cell height, (lateral) cell diameter) and dimension-related parameters (cell surface area and volume) of individual chondrocytes during the proliferative and late hypertrophic activity phases within growth plate cartilage, derived from rats of different ages, are presented in Table 2. During growth acceleration (compare data for 21- and 35-day-

TABLE 1. Growth plate height (of proximal tibia) related to growth rate and body weight in different age groups of rats

Animal age...	21 days	35 days	80 days
Body weight (g)	40·3 (6·0)	124 (2·5)	299 (1·4)
Longitudinal growth rate (μm/day)	276 (2·3)	330 (1·3)	85 (2·3)
Height of			
total growth plate (μm)	653 (2·7)	583 (8·1)	216 (6·7)
RZ	41 (12·6)	42 (8·8)	19 (9·9)
PZ	226 (5·2)	171 (5·0)	78 (8·2)
HZ	386 (2·4)	370 (8·1)	119 (7·5)

Values represent means for six animals. Coefficients of error (calculated between the six animals) are given in parentheses (in %).
Abbreviations used: RZ = resting zone; PZ = proliferative zone; HZ = hypertrophic zone.

TABLE 2. Stereologic estimators for individual chondrocytes

	Proliferative activity phase			Hypertrophic* activity phase		
Animal age...	21 days	35 days	80 days	21 days	35 days	80 days
Cell height (μm)	8·1 (8·4)	9·6 (6·6)	8·2 (6·3)	31·2 (6·6)	38·5 (5·3)	18·2 (4·7)
Cell diameter (μm)	22·4 (5·0)	19·0 (4·3)	19·3 (5·8)	29·9 (4·4)	25·6 (3·6)	28·9 (3·8)
Cell surface area (μm^2)	1030 (4·2)	857 (3·1)	852 (10·7)	3971 (3·6)	3440 (5·4)	1959 (5·0)
Cell volume (μm^3)	2088 (4·5)	1790 (4·7)	1576 (8·9)	19975 (4·4)	17400 (6·2)	7576 (6·5)
Matrix volume per cell (μm^3)	3470 (5·5)	2830 (7·6)	3710 (8·9)	7970 (8·1)	7730 (8·7)	7660 (5·6)

Mean values for six animals are represented. Coefficients of error (calculated between the six animals) are given in parentheses (in %).
* Values refer to chondrocyte activity within the lower hypertrophic zone (i.e. refer to the final activity phase).

old rats in Table 2), the 20% increase in growth rate (cf. Tables 1 and 4) could be accounted for almost exclusively by an increase in the final cell height (by 23%, from 31·2 to 38·5 μm, $P < 0{\cdot}02$; cf. Table 2) achieved during hypertrophy, the duration of which remains constant (cf. Tables 3 and 4). Since the final lateral cell diameter achieved during this activity phase is reduced (by 14%, from 29·9 to 25·6 μm,

TABLE 3. Columnar cell pool sizes and kinetics within the rat proximal tibial growth plate

Animal age...	21 days	35 days	80 days
Number of proliferative cells/column*	27 (6·3)	18 (7·2)	9 (13·1)
Number of hypertrophic cells/column	17 (5·0)	15 (4·9)	8 (5·2)
Cell turnover/column per 24 h	8 (7·2)	8 (3·7)	4 (5·0)
Cycle time for a proliferating cell (h)	81 (11·4)	54 (9·5)	54 (14·3)
Duration of hypertrophic phase (h)	51 (5·7)	45 (8·7)	48 (4·1)

Mean values for six animals are represented, and due to the necessarily approximate nature of the calculations (cf. Cruz-Orive & Hunziker, 1986) they are rounded off to the nearest integer. Coefficients of error (calculated between the six animals) are given in parentheses (in %).

* This value is equivalent to the so-called (columnar) 'growth fraction'.

TABLE 4. Relative* changes (%) in various cell and column activity parameters with respect to growth acceleration and deceleration

Age groups compared...	21–35 days (representing growth acceleration)	35–80 days (representing growth deceleration)
Longitudinal growth rate (μm/day)	+20	−75
Final cell height achieved	+23	−53
Final (lateral) cell diameter achieved	−14	+13
Final cell volume achieved	−13	−56
Final matrix volume per cell	0	0
Cell cycle time of a proliferating cell	−33	0
Duration of hypertrophic activity	0	0
Number of cells produced/eliminated (per column per day)	0	−50
Columnar 'growth fraction' (i.e. number of proliferating cells/column)	−33	−50

* Relative change $= \frac{b-a}{a} \times 100\%$.

$P < 0{\cdot}03$; Table 2), physiological growth acceleration appears to be accomplished solely by cell-shape modelling, i.e. phenotype modulation. No complementary increases in either final cell volume or matrix production per cell were found (Tables 2 and 4). Indeed, the final net matrix volume produced per cell remains constant (i.e. does not differ significantly between 21- and 35-day-old rats), and the final cell volume achieved is even reduced (by 13%, Table 4; $P < 0{\cdot}03$).

The rate of cell production (or elimination) per column (i.e. turnover in the longitudinal direction) also remains unchanged, despite a reduction in the calculated value for cell cycle time for proliferating cells (i.e. by 33%, from 81 to 54 h; cf. Tables 3 and 4), a change which would be expected to accompany an increase in cell turnover. The decrease in mean cell cycle time is, however, offset by a simultaneous and corresponding decrease in the columnar 'growth fraction', namely, the number

of proliferating chondrocytes per column (by 33%, from twenty-seven to eighteen cells, cf. Tables 3 and 4).

Although the various estimators describing the dimensions and dimension-related parameters of individual *proliferating* chondrocytes (Table 2) also increase and decrease corresponding to the trends recorded for hypertrophic cells during growth acceleration, none of these values differ significantly. Significant changes in cell performance during increase in linear bone growth rate are thus achieved exclusively during the hypertrophic activity phase (see Figs 2 and 3).

Reductions in final cell height, volume and surface area achieved during hypertrophy all appear to contribute to decreases in linear bone growth rate (compare data for 35- and 80-day old rats in Tables 2 and 4; Figs 1 and 3). And as found during growth acceleration, estimates for cell activity do not differ significantly in the proliferative phase. Hypertrophic cell activities thus again contribute most significantly to the regulation of linear growth, but during growth deceleration, changes in both final cell height and volume play major roles (with relative reductions of 53 and 56%, respectively, cf. Table 4).

During growth deceleration, the rate of cell production (or elimination) per column is reduced (by 50%, from eight to four cells per column per day, cf. Table 3). This change is effected by a corresponding (i.e. 50%) decrease in the columnar 'growth fraction' (i.e. from eighteen to nine cells, cf. Tables 3 and 4), since the cell cycle time for proliferating cells remains unchanged.

As during growth acceleration, the final net matrix volume produced per cell remains constant (i.e. no significant changes are measured during growth deceleration, cf. Tables 2 and 4).

DISCUSSION

Longitudinal bone growth depends primarily upon the co-ordinated activities of chondrocytes within growth plate cartilage, and hence the rate at which this process occurs will reflect specific changes in one or several parameters characterizing chondrocyte production and development. It was thus the aim of the present study to gain an insight into the physiological mechanisms adopted by chondrocytes for longitudinal growth modulation, and to determine how these specific activites are attuned, in relation to this phenomenon, during ageing.

During the rat pre-pubertal growth spurt (35 days; Kember, 1973, 1983), longitudinal growth rate increases of approximately 20% are accounted for almost exclusively by an increase in mean cell height during hypertrophy (by 23%, $P < 0{\cdot}02$). The simultaneous, but relatively smaller, changes in lateral cell diameter and final cell volume attained during this terminal stage imply that growth acceleration relies solely upon cell-shape modelling (cf. Figs 2 and 3). In consequence of this finding, it can only be assumed that regulation of growth by this mechanism is the most efficient. Indeed, the energy requirements for phenotype modulation are likely to be conservative compared with those necessary for effecting increases in cell volume, for instance, which is achieved by active transport of fluid and electrolytes across the plasma membrane against the high osmotic pressure within the matrix (for discussion see Hunziker *et al.* 1987).

Although cell phenotype is classically maintained and modulated by components of the cytoskeletal system, chondrocytes are not well endowed with these structures (Fig. 3), suggesting that a different mechanism of control may be operative. Indeed, recent experiments carried out with chondrocytes *in vitro* indicate that their shape is maintained more or less independently of cytoskeletal components (Benya, Brown

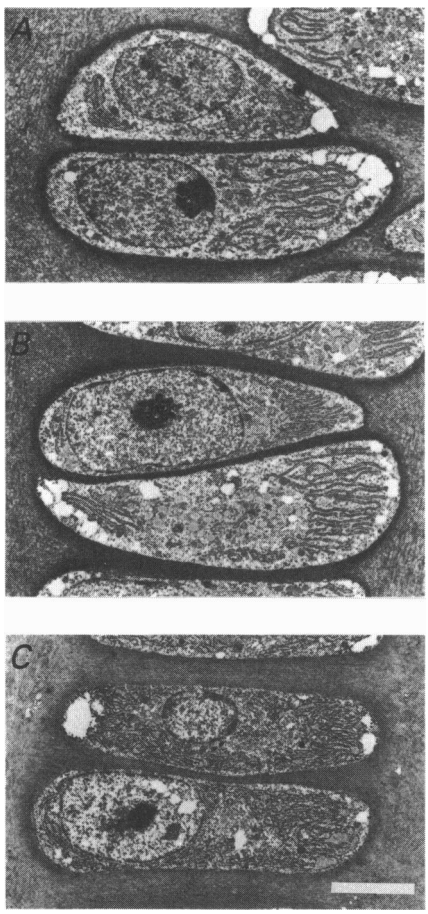

Fig. 2. Low-power electron micrographs (each at a magnification of ×2200) of growth plate chondrocytes in the proliferating phase of a 21-day-old (*A*), 35-day-old (*B*) and 80-day-old (*C*) rat. The reader should compare the size of these cells with those of the lower hypertrophic zone in Fig. 3, which are illustrated at *identical* magnifications. Bar = 5 μm.

& Padilla, 1988). It seems likely that chondrocyte shape and volume are modified subsequent to and not prior to changes in the pattern of matrix degradation and synthesis, albeit that the initial signal for matrix remodelling is cell-derived (see Fig. 4). The basis for such a mechanism lies (1) in the nature of the matrix which despite being elastic and compressible is also stiff, and thus provides a firm, supportive coat around the chondrocyte, and (2) in the intimate contact existing between the chondrocyte plasma membrane and the pericellular matrix (Hunziker, Herrmann & Schenk, 1983; Hunziker & Schenk, 1984). It is envisaged that the chemical

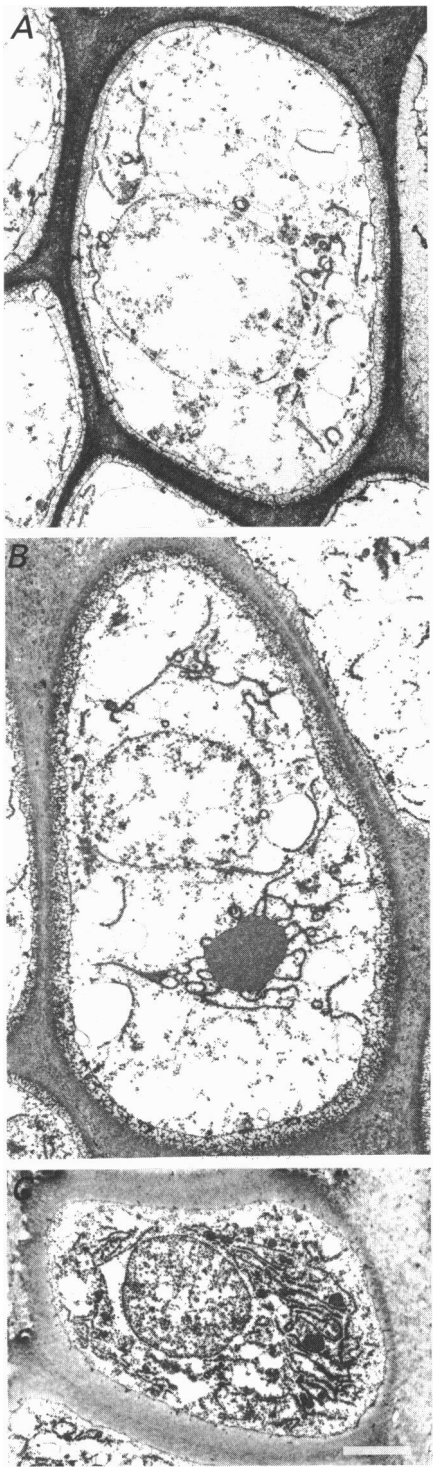

Fig. 3. Low-power electron micrographs of growth plate chondrocytes in the lower hypertrophic zone of a 21-day-old (*A*), 35-day-old (*B*) and 80-day-old (*C*) rat, shown at *identical* magnifications ($\times 2200$). Bar = 5 μm.

interactions established between plasma membrane and pericellular matrix rim are maintained during matrix remodelling (with focal disruption and re-establishment at points of matrix degradation being achieved at the molecular level), such that the cell contours follow the mould formed by the matrix rim. Such movement could, of course, not be entirely passive, and would necessitate co-ordinated surface membrane synthesis (at points of matrix degradation) and a counteracting increase in fluid and electrolyte transport into the cytoplasm (which even under 'resting' conditions is continually activated) in order to maintain cell volume and electrolyte balance against the high osmotic and swelling pressure of the matrix. Morphological inspection of chondrocytes *in situ* offers support for a high degree of co-ordination between matrix remodelling and chondrocyte shape change (Figs 2 and 3).

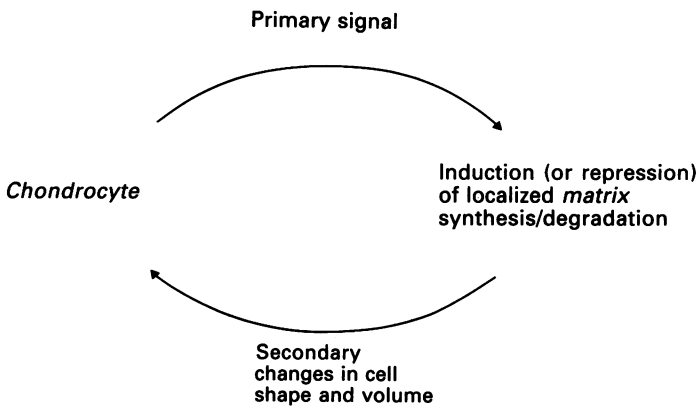

Fig. 4. Hypothetical mechanism of chondrocyte phenotype regulation. The chondrocyte regulates its shape and volume indirectly via changes in matrix degradation and synthesis, the feedback relationship between cell and matrix being such that perfect synchrony between the two is maintained during remodelling.

Individual chondrocytes perform their activities within the cell columns that they occupy, these representing the functional units for longitudinal bone growth. Under physiological conditions, cell production rates are usually regulated by changes in the 'growth fraction' (corresponding to the proliferating cell pool within a column in this investigation) with cell cycle times remaining constant (Steel, 1977; Kember, 1978; Pardee, Dubrow, Hamlin & Kletzien, 1978). Such a pattern is followed during growth deceleration, but not during growth acceleration. In the latter situation, the unexpected reduction in the columnar 'growth fraction' (from twenty-seven to eighteen proliferating cells per column, Table 3) is compensated for by a shortening of the cell cycle time (from 81 to 54 h; Table 3), such that the basic linear production rate of cells (eight chondrocytes per column per day) remains unchanged. The cell cycle time of 54 h for proliferating chondrocytes in 35- and 80-day-old rats is close to that found by Kember (1983) and Walker & Kember (1972*a*) using an alternative method. The anomaly presented by that for proliferating chondrocytes in 21-day-old rats (i.e. 81 h) is difficult to justify, except by inferring that subpopulations of proliferating cells with differing cell cycle times (for example one with the expected

54 h cycle time and another with a much longer one) do indeed exist (Walker & Kember, 1972*a*, *b*; Kember, 1978, 1983).

The regulatory mechanisms implicated for growth deceleration are more complex than those involved during growth acceleration. The kinetic regulation follows the usual pattern, the decrease in cell turnover (by 50%, Table 4) being achieved by a reduction in the columnar 'growth fraction', with cell cycle time remaining constant (Kember, 1978). In addition, both phenotype modulation (with final cell height and diameter reductions of 53 and 13%, respectively, Table 4) and cell volume regulation (with a final reduction of 56%, Table 4) appear to be involved to a significant degree.

During both acceleration and deceleration of linear growth, changes in hypertrophic cell activities (in addition to alterations in columnar cell proliferation rate during growth deceleration) appear to play an important regulatory role. These results are somewhat surprising, since it has been generally assumed that linear growth is modulated principally by changes in proliferation activity. A possible reason for the importance of cell hypertrophy is that it is a much faster and more efficient means to rapidly prolong the columnar units than is the addition of new chondrocytes. Although the energy requirements necessary for active transport of fluid and electrolytes into cells during hypertrophy (Figs 2 and 3; see Hunziker *et al.* 1987 for discussion) may be high, the expenditure of energy necessary for producing new cells for an identical columnar volume increase is likely to be much higher. This may be illustrated by example. In 35-day-old rats, a columnar volume increase of $\sim 15610\ \mu m^3$ (i.e. terminal chondrocyte volume ($17400\ \mu m^3$) minus end-proliferating cell volume ($1790\ \mu m^3$), Table 3), is achieved by hypertrophy of a single chondrocyte. If a similar volume increase was to be achieved by cell proliferation, approximately nine cells would have to be produced. Moreover, a proliferating chondrocyte needs ~ 54 h (= cell cycle time, Table 3) to duplicate its own volume, whereas during hypertrophy a corresponding volume increase would be achieved within a period (Δt) as short as 5 h (Δt = duration of hypertrophic activity, i.e. 45 h, divided by the number of proliferating chondrocytes equivalent to one terminal chondrocyte in volume, i.e. nine cells). Hypertrophy (including the process of phenotype modulation) thus appears to be a much more efficient mechanism for effecting columnar linear growth than cell proliferation alone would be. These two main processes (i.e. cell proliferation and hypertrophic cell volume increase) do not, however, bear a constant relationship to one another under the various growth rate conditions, nor indeed is either parameter linearly related to longitudinal growth rate. Matrix production by chondrocytes is generally assumed to increase or decrease with corresponding changes in growth rate. From the results presented in this report it is clear, however, that by the end of its life cycle, a hypertrophic chondrocyte has procured no net increase or decrease in its associated matrix volume, either during growth acceleration or deceleration (cf. Table 2; matrix volume per cell, hypertrophic activity phase). It should be noted that our data are stereologic estimators (i.e. morphologically measurable parameters), and an increase in matrix volume could, theoretically, be a manifestation of a dilution effect during the hypertrophic activity phase. However, quantitative autoradiography of cartilage matrix proteoglycans, by measurement of [^{35}S]sulphate incorporation, has excluded this possibility (unpub-

lished data). Although there is no net increase in matrix production during growth acceleration, the total mass produced per cell in the course of hypertrophy, including that subsequently degraded to accommodate for phenotype modulation, could of course play a significant role in *effecting* but not in *regulating* longitudinal growth (since it does not contribute directly to acceleration (or deceleration) of this process by column prolongation). The functions of matrix most likely involve space filling between cells to compensate for changes in height, diameter and volume, thus helping to maintain the highly anisotropic columnar tissue organization during linear growth. Hence, they are related to retaining the biomechanical properties (Buckwalter, 1983; Hunziker & Schenk, 1984) of growth plate cartilage, and in helping to integrate chondrocytes in a highly ordered fashion into this tissue.

The duration of the hypertrophic phase ($\sim$ 48 h) was found to remain remarkably constant, irrespective of animal age or growth rate. This finding confirms the importance of phenotype modulation as a regulatory mechanism during longitudinal growth acceleration, since otherwise one would have expected the observed increase in hypertrophic cell height to be achieved by an increase in hypertrophic phase duration. It suggests, moreover, that the preparative period required for matrix mineralization cannot be speeded up or slowed down. Without the induction of this process, vascular invasion, and hence longitudinal bone growth, would be impossible (in mammalian growth plates). It thus seems likely that this preparative period dictates the duration of the hypertrophic phase. In analogy to this, it has previously been found that unmineralized bone matrix (osteoid) needs to be deposited extracellularly for a minimum period of time (2–3 days in the woven bone and approximately 10 days in the lamellar bone of small rodents) before mineralization can begin (Schenk, Hunziker & Herrmann, 1982).

Quantitative assessment of longitudinal bone growth is frequently based upon measurement of bulk parameters (Nevo & Laron, 1979; Seinsheimer & Sledge, 1981; Phillips & Weiss, 1982) such as growth plate height ('tibia test'; Greenspan *et al.* 1949; Phillips & Weiss, 1982), cell proliferative activity (determined by [^{3}H]thymidine incorporation into chondrocytes; Chochinov & Daughaday, 1976) or matrix production (evaluated by measurement of [^{35}S]sulphate incorporation into matrix components, i.e. the so-called 'sulphation factor'; Dziewiatkowski, 1964; Daughaday, Hall, Raben, Salmon, Van den Brande & Van Wyk, 1972). Data derived from such determinations may, however, yield a misleading picture, since each of these parameters represents a compilation of growth responses, not all of which contribute to bone elongation. The misconceptions that may consequently arise are well illustrated by the following examples.

It is apparent from Table 5 that modulations in growth rate and growth plate height do not occur in parallel; indeed, the direction of these changes (i.e. whether increasing or decreasing) is not even consistent between the two parameters. Although both decrease (but not proportionately) during growth deceleration (between 35 and 81 days), an acceleration in growth rate (between 21 and 35 days) is accompanied by a decrease in growth plate height.

Cell proliferation rate and matrix production are usually determined per volume (or weight) of tissue. Hence, in order that the reader may identify more closely with the implications of our findings, we have here related our data for these two

parameters to a unit volume (i.e. 1 mm^3) of growth cartilage tissue (rather than per column (for cell proliferation) or per cell (for matrix production)). When cell proliferation rate is expressed in this way (see Table 5), it may be seen that an acceleration of growth by 20% (between 21 and 35 days) is accompanied by a 55% increase in cell proliferation, whereas a 75% decrease in growth rate (between 35 and 80 days) is accompanied by an 18% increase in cell proliferation. Moreover, when

TABLE 5. Comparison between relative changes (%) in growth rate and so-called 'bulk' parameters: cell proliferation, matrix production and growth plate height

Rat age group comparisons	Change in growth rate	Growth plate height (μm)	Cell proliferation* (/ 24 h per mm^3 tissue)	Matrix production† (/ 24 h per mm^3 tissue)
21–35 days	+20	−11	+55	+50
35–80 days	−75	−63	+18	+16
21–80 days	−69	−67	+81	+74

* Absolute values determined as the product of the number of cells produced per column per 24 h and the number of columns per mm^3 of tissue.

† Absolute values determined as the product of cell turnover (calculated per mm^3 of tissue per 24 h) and final net matrix volume per cell.

comparing 21- and 80-day-old animals, representing an overall growth deceleration of 69%, cell proliferation increases by 81%. The increased proliferative cell activity measured per unit volume of tissue during growth deceleration, is attributable to an increase in the number of cell columns, and hence in the proliferating cell pool, occurring as a result of lateral growth plate expansion (Heřt, 1972). This more than compensates for the decrease in columnar cell height. A similar picture is revealed for measurements of [^{35}S]sulphate incorporation into cartilage matrix (see Table 5).

The chondrocyte activities revealed as being of particular importance in governing changes in longitudinal growth, most particularly cell phenotype modulation, once again (see Hunziker *et al.* 1984, 1987) provide evidence for the highly active state of the hypertrophic cells, and point to their singular importance in mineralization induction.

The authors wish to thank W. Graber for his expert technical assistance, Ceri England for her English correction of the manuscript and Luis M. Cruz-Orive for his advice concerning stereological procedures. This work was supported by the Swiss National Science Foundation, grant No. 3.058-0.84, and the AO/ASIF-Foundation, Switzerland.

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