PHYSEAL DISTRACTION AND CELL PROLIFERATION IN THE GROWTH PLATE

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We studied the cellular response to physeal distraction in the growth plates of skeletally immature rabbits. We used a new method of labelling and detection of proliferating cells with bromodeoxyuridine (BUdR) and an anti-BUdR antibody.

The application of an external fixator but no distraction force produced no changes in the growth plates. After five days of distraction at a maximum force of 20 N, the growth plate became thicker, mainly because of an increase in the number of hypertrophic chondrocytes, but there was no evidence of increased cell proliferation. Recent fractures were seen at the junction of growth plate and metaphysis but the increase in bone length was insignificant. After ten days of distraction at the same maximum force, the chondrocyte columns had become disorganised and cell proliferation was significantly decreased. There was an increase in bone length due to distraction of the fracture gap.

In this model, physeal distraction did not stimulate cell proliferation, but actually inhibited it. The apparent increase in growth-plate thickness produced by distraction is not due to increased cell production, but results from inhibition of endochondral ossification and the consequent accumulation of hypertrophic chondrocytes. Any growth after distraction depends on the ability of growth-plate chondrocytes to divide. The decrease in proliferative activity which we found after ten days of distraction suggests the need for caution in the use of such procedures in young children.

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Physeal distraction is used for limb lengthening or correction of deformities but there is concern about the growth potential of the distracted physis. Ring (1958) showed that distraction across the physis causes rupture through the hypertrophic zone, and others have confirmed that lengthening is achieved by distraction of the fracture gap (Ilizarov and Soĭbel'man 1969; Monticelli and Spinelli 1981a,b). Such fractures require high force levels (Jones et al 1989; Kenwright, Spriggins and Cunningham 1990) and most evidence suggests that growth may be inhibited either partially or completely after distraction (Hert 1969; Fishbane and Riley 1978; Monticelli and Spinelli 1981a; Connolly et al 1986). It has been shown in an animal model that lower forces or slower rates of distraction reduce the risk of subsequent longitudinal growth disturbance (de Pablos, Villas and Canadell 1986), and it has been suggested that low forces or slow distraction increase length by causing hyperplasia of the growth plate without fracture (De Bastiani et al 1986a; Wilson-MacDonald et al 1990).

This type of overgrowth without fracture is called chondrodiatasis (De Bastiani et al 1986b). Sledge and Noble (1976) also showed that slow rates of distraction with a constant force could increase bone length with or without associated fracture; they found increased cellular activity in the growth plate. Elmer et al (1992), however, were able to distract the physis in the rabbit at a similar slow rate with no evidence of altered growth-plate activity.

The effect of slowly progressive distraction on cellular activity in the physis is uncertain, both during and after completion of the lengthening. The fundamental mechanism of longitudinal bone growth is the production of new chondrocytes in the proliferative zone of the growth plate (Kember 1983). This increases the number of cells, the size of the cells and the volume of matrix production, all of which contribute to lengthening.

The analysis of cell proliferation has been limited by the techniques available; traditional studies have used mitotic, stathmokinetic, or autoradiographic methods (Kember 1983). We have used a new immunohistochemical approach after distraction at a force level which in previous studies led to plate hypertrophy either with or without fracture (Sledge and Noble 1976; Kenwright et al 1990).

We hypothesise that such distraction regimes may affect cell production in the proliferative zone and
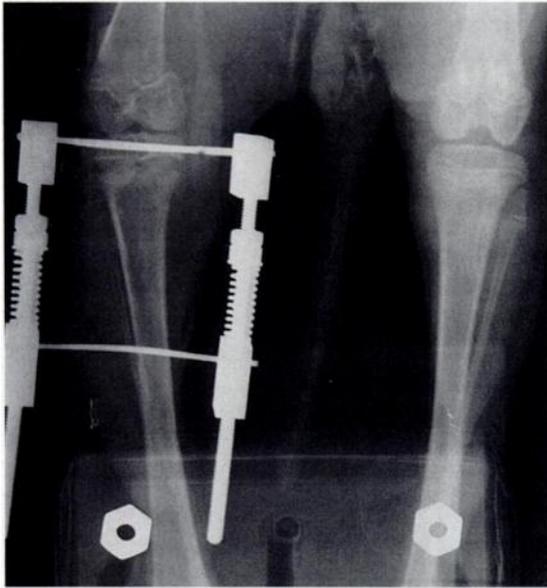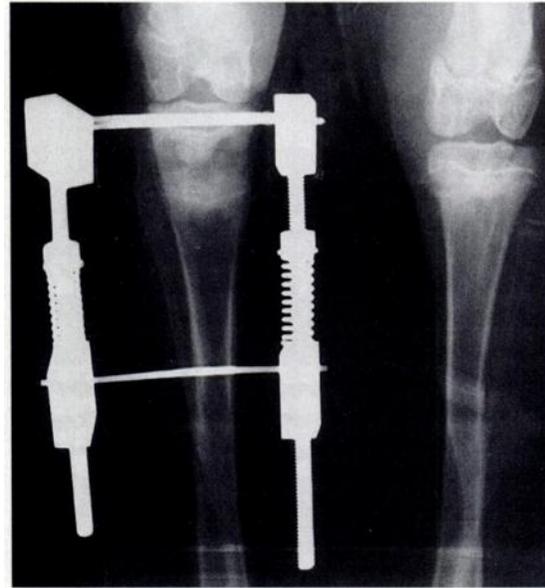
Fig. 1a   Fig. 1b

Radiographs of the control and experimental limbs after five days of distraction (a) and ten days of distraction (b).

subsequent ossification of the hypertrophic zone of the physis.

MATERIALS AND METHODS

Six-week-old male New Zealand white rabbits with considerable growth potential (Masoud et al 1986) had their right proximal tibial physis distracted by a bilateral-frame external fixation system (Fig. 1). The main fixator bar was loaded by a spring which delivered the distraction force. The springs were calibrated before use; each showed a linear relationship between its length and the force to which it was subjected. Pairs of springs of identical mechanical behaviour were used for each rabbit.

A single transverse skin incision was made on the medial aspect of the knee and the line of the proximal tibial growth plate identified by minimal dissection. Two 1.1 mm Kirschner wires were passed through the bony epiphysis and one distally through the diaphysis. All three were attached to the external fixator. Three groups of experimental animals were used:

Group A (5 controls). External fixation was applied but without distraction.
Group B (5). A maximum distraction force of 20 N was achieved each day for five days.
Group C (8). A maximum distraction force of 20 N was achieved each day for ten days.

The external fixators were adjusted daily, at the same time, to apply a maximum force of 20 N. On completion of the experimental period animals were injected intravenously with 40 mg/kg of bromodeoxyuridine (BUdR) which is a thymidine analogue. This was given as an 8 mg/ml solution in sterile normal saline. One hour later the rabbits were killed with an overdose of anaesthetic.

BUdR was given at the same time of the day in all animals to eliminate the known diurnal variation in DNA labelling indices (Kember 1983).
Post-mortem studies. Tibial lengths were measured at the beginning and end of the experiment from radiographs taken under standard conditions, with the animal under sedation and the leg attached to a jig. Measurements were made from the tibial spine to the medial malleolus using a vernier calliper; corrections were made for magnification. At the end of the experiment tibial lengths were measured directly on the dissected bones.

The proximal end of each tibia was bisected in the sagittal plane and cut into 3 mm slices. These were fixed in Carnoy's fluid for three hours, decalcified in 14% ethylenediamine tetra-acetic acid and embedded in paraffin. Sections were stained with haematoxylin and eosin with toluidine blue, and with phosphotungstic acid haematoxylin (PTAH) for demonstration of fresh fibrin. An eyepiece graticule previously calibrated with a stage micrometer was used to measure the total vertical height of growth-plate cartilage from the epiphyseal bone plate to the last intact transverse septum in the hypertrophic zone. Twenty such observations were obtained on each slide and recorded as mean and standard deviation.

BUdR is incorporated by cells in the S-phase of the cell cycle (DNA synthesis); the number and distribution of BUdR-labelled cells are therefore representative of the proliferating cell population (Gratzner 1982). They can be localised by the use of monoclonal antibodies (Gratzner 1982; Magaud et al 1989). This method has been shown to be valid in detecting S-phase cells in decalcified paraffin-embedded (Apte 1990) and undecalcified plastic-embedded sections of skeletal tissues (Apte and Puddle 1990). The BUdR method provides the same data as those

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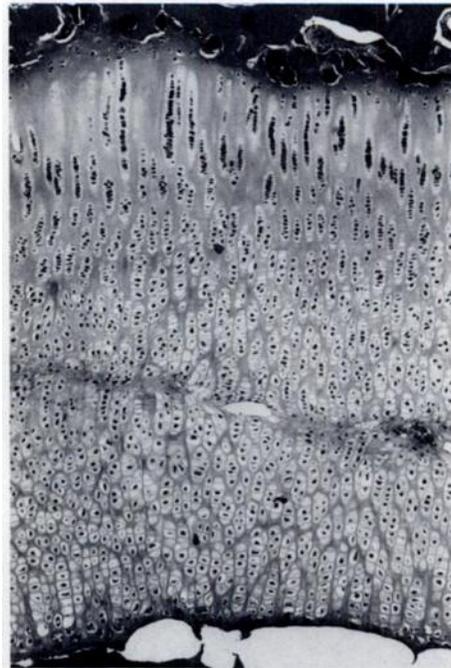
Fig. 2b
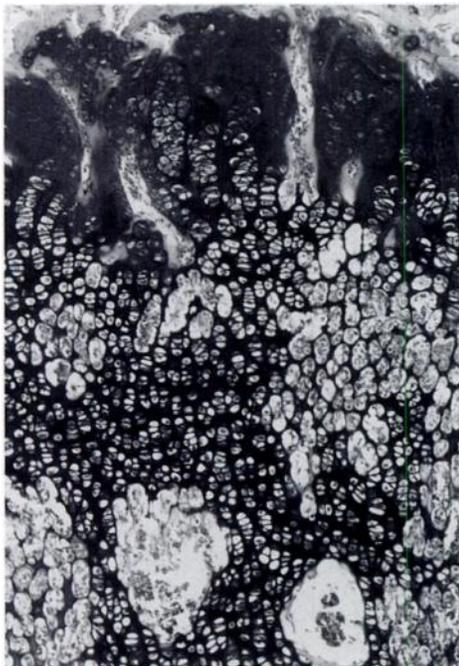
Fig. 2c

Figure 2a – An undistracted growth plate stained with haematoxylin and eosin. Figure 2b – Growth plate after five days of distraction, stained with PTAH. There is an increase in the mass of cartilage caused by an increase in the number of hypertrophic chondrocytes. A fracture gap containing fibrin is seen at the bottom of the figure. Figure 2c – A growth plate after ten days of distraction, stained with toluidine blue. The increased mass of hypertrophic chondrocytes is in irregular columns and blood vessels are present. There was no measurable increase in the size of the proliferating columns ($\times$80).

produced by tritiated thymidine autoradiography, but is more rapid and is radiation-free. Another advantage is that high magnification and grain-counting (Elmer et al 1992) are not required to identify labelled cells: the new method provides a 'bird's-eye view' of cell proliferation in relation to the tissue architecture. For cell-proliferation studies we used paraffin sections 5 μm thick immuno-stained with the anti-BUdR monoclonal antibody Bu20a (Magaud et al 1989) as described by Apte (1990).

The proliferative zone was defined as the zone which includes all cells between the most epiphyseal and the most metaphyseal labelled cells; and its size as the number of cells in this zone.

We assessed the size in terms of the distribution of proliferating cells per column by construction of labelling profiles for the growth plate (Kember 1983). The percentage of BUdR-labelled nuclei (the BUdR labelling index) was determined from a count of at least 1000 nuclei in the proliferative zone.

We determined the statistical significance of the difference between left (control) and right (distracted) growth plates by using the Wilcoxon signed-rank test for matched pairs.

RESULTS

Group A (controls). One animal was excluded because of infection. There were no significant differences in the tibial lengths, histology, growth-plate height or BUdR immunostaining between the right and left tibiae in the remaining four animals in this group.

Group B (5-day distraction). One animal died. Four distracted growth plates and controls from the opposite tibia were studied.
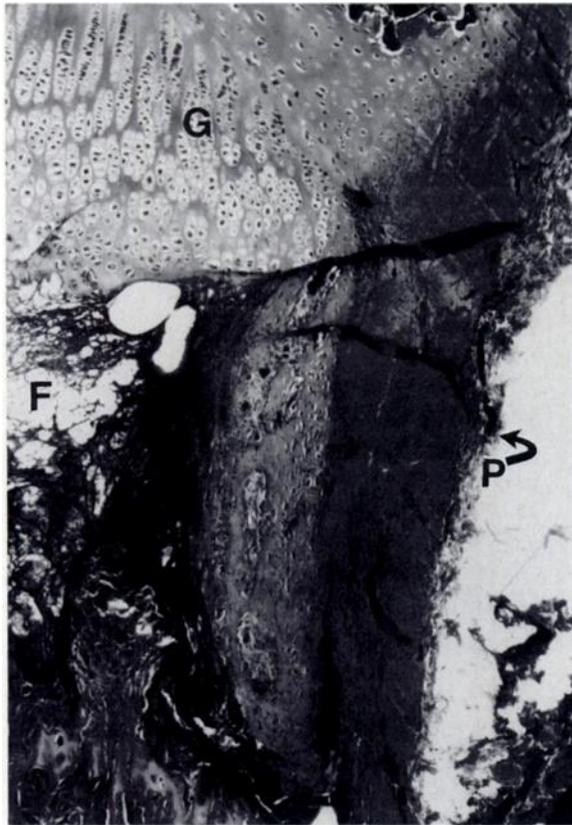
Fig. 3

The periphery of a growth plate after five days of distraction, stained with PTAH. In contrast to the central zone of the plate, shown in Figure 2b, there is no thickening at the periphery. The growth plate (G) has undergone a traumatic separation from the perichondrium and metaphysis. Fresh fibrin (F) fills the fracture gap. The prominent fibrous periosteum (P) has remained intact (×100).

Radiology. In distracted limbs, the proximal tibial growth plate showed an increase in thickness (Fig. 1a) although there was no statistically significant increase in tibial length.

Histology. The increase in thickness of growth-plate cartilage was clearly visible to the naked eye and was confirmed by histological examination (p < 0.05). It was most obvious in the centre of the plate (Figs 2 and 3). Three of the four specimens showed clear evidence of recent fracture: PTAH-stained sections revealed fresh fibrin and haemorrhage within the fracture gap (Figs 2 and 3). The fractures ran through the junction between the hypertrophic zone and the metaphysis, but one specimen showed an additional fracture line through the proliferative zone of the growth plate. The cartilage mass consisted predominantly of hypertrophic cells arranged in columns, but the resting and proliferative zones appeared to be unchanged compared with those in the plate from the control limb. One specimen showed necrosis within the proliferative and reserve zones. The structures within the perichondrial groove of Ranvier were not disturbed and the outer fibrous layer of the periosteum remained intact; the growth cartilage had pulled away from the

inner aspect of the bone in the region of the perichondrium (Fig. 3).

BUdR immunostaining. In three animals the size of the proliferative zone and the BUdR labelling index on the distracted side were similar to those on the control side (Table I). Despite the considerable increase in cartilage mass on the distracted side, the number of BUdR-labelled cells was not increased in each column (see Fig. 4b) nor was the labelling index (Table I). In the remaining specimen there was no BUdR uptake in the proliferative zone in its central two-thirds (the area of increased cartilage volume) although labelling was seen peripherally. No anomalous cell proliferation was seen in either the reserve zone or the hypertrophic zone.

Group C (10-day distraction). One rabbit in this group was excluded because of local infection.

Radiology. There was a statistically significant increase in bone length on the distracted side (1.4 to 7.4 mm; p < 0.01) with a transverse zone of increased lucency just distal to the growth plate, representing the zone of fracture distraction (see Fig. 1b).

Histology. As in group B there was an increase in the thickness of the distracted growth plate (p < 0.01). This was irregular, with a greater increase in the centre than at the periphery. All seven distracted growth plates showed an irregular arrangement of cell columns in the proliferative and hypertrophic zones (Fig. 2c). One had areas of necrosis within the proliferative zone; the matrix in this region was less basophilic on haematoxylin staining, and stained poorly with toluidine blue. The perichondrial groove was unaffected, as in group B, and the periosteum was intact.

BUdR immunostaining. BUdR immunohistochemistry showed that the proliferative zones of the distracted growth plates were smaller than those in the control growth plates and the distracted growth plates of group B. There was a statistically significant decrease in the labelling index, reflecting the decrease in cell production in this layer (Fig. 4; Table I). There was no anomalous cell proliferation in either the reserve zone or the hypertrophic zone. No BUdR uptake was seen in the cartilage islands which were found in the metaphysis.

DISCUSSION

The maintained axial tension of 20 N for four to five days, approximately equivalent to body-weight (Masoud et al 1986), produced a Salter-Harris type-I fracture across the upper tibial growth plate in young rabbits. The exact moment of fracture was not defined, but the site was usually at the junction between the growth plate and the metaphysis, suggesting that this interface is a weak link. Similarly, at the periphery, the growth cartilage was torn away from the subperiosteal and perichondrial bone, leaving the perichondrial ring and periosteum intact.

Our histological findings were similar to those reported by previous investigators. There was an irregular
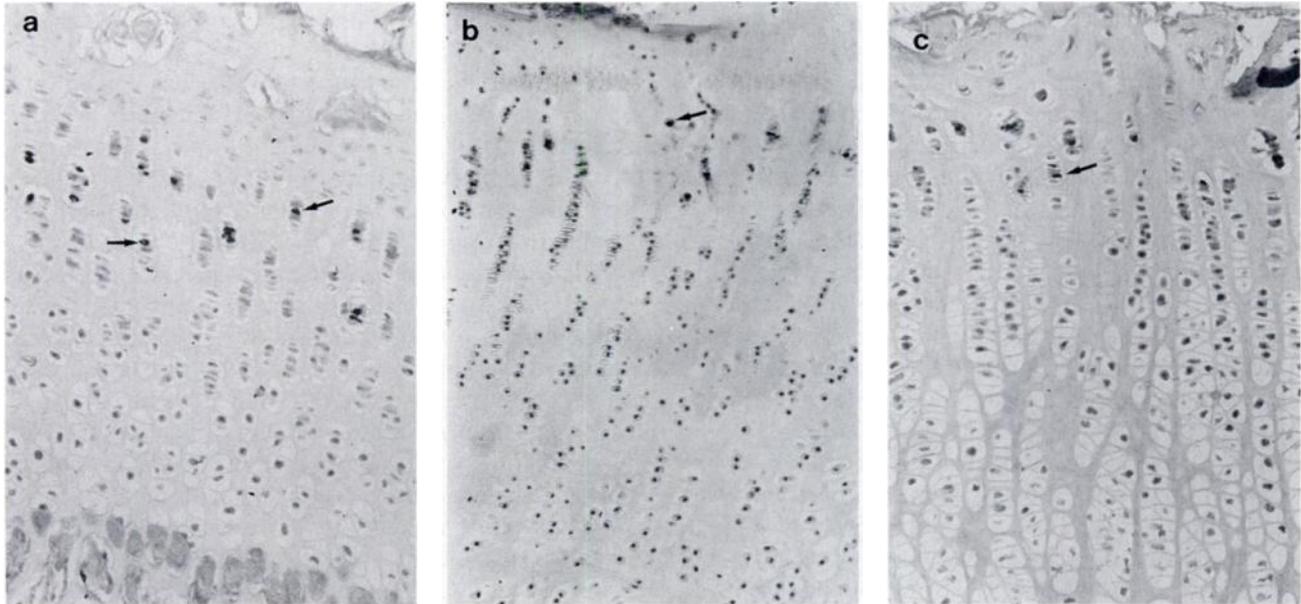
Fig. 4a   Fig. 4b   Fig. 4c

Sections of a proximal tibial growth plate. Proliferating cells are labelled with BUdR, showing nuclear deposits of diaminobenzidine (arrows). The counterstain is haematoxylin. Figure 4a – Control, undistracted growth plate (×200). Figure 4b – After five days of distraction there is an increase in the height of the growth plate compared with that in Figure 4a, but most of the cells are hypertrophic chondrocytes, and the zone of proliferating chondrocytes defined by BUdR staining is similar in both figures (×100). Figure 4c – After ten days of distraction, the zone of BUdR-labelled cells is narrower than in the other figures (×200).

Table I. Quantitative results of BUdR immunostaining in distracted and control growth plates

		Labelling index*		Size of proliferative zone*	
Group	Number	Distracted	Control	Distracted	Control
A (control)	1	21.5	23.2	20	21
	2	21.9	21.8	25	22
	3	24.1	22.4	21	23
	4	19.7	20.9	20	19
		Mean = 21.8	Mean = 22.1	Mean = 21.5	Mean = 21.25
		Not significant		Not significant	
B (5-day distraction)	1	21.2	25.2	25	20
	2	21.3	22.1	23	21
	3	0	22.3	0	20
	4	23.2	20.5	25	19
		Mean = 16.4	Mean = 22.5	Mean = 18.25	Mean = 20
		Not significant		Not significant	
C (10-day distraction)	1	19.7	18.4	20	17
	2	4.4	20.2	10	20
	3	4.6	19.7	22	21
	4	8.3	21.2	11	17
	5	1.1	18.9	14	19
	6	13.0	22.4	19	21
	7	17.5	28.8	19	21
		Mean = 9.8	Mean = 21.4	Mean = 16.4	Mean = 19.4
		$p < 0.01$		Not significant	

* see text

increase in thickness of the growth plate in all specimens subjected to distraction, most obvious in the central region. This thickening has been described under conditions of low force or low rate of distraction, sometimes with fractures and sometimes without (Sledge and Noble 1976; Peltonen et al 1988; Spriggins et al 1989; de Pablos and Canadell 1990; Wilson-MacDonald et al 1990). We found that this increase in thickness occurred in the hypertrophic zone, but others have reported it in both the proliferative and the hypertrophic zones (Sledge and Noble 1976; De Bastiani et al 1986b).

We believe that the most probable cause for the accumulation of hypertrophic chondrocytes was the induction of metaphyseal ischaemia. Trueta and Amato (1960) showed that the occlusion of metaphyseal vessels caused hypertrophic chondrocytes to accumulate in a

pattern very similar to that seen in the distracted growth plates in our study. Noguchi, Yamaguchi and Sugioka (1994) have shown that interruption of metaphyseal blood flow results not only in lack of calcium in hypertrophic chondrocytes, but also in inhibition of cartilage resorption. As reported by Elmer et al (1992), we occasionally found necrosis within the growth plate, and after ten days of distraction, we found islands of hypertrophic chondrocytes within the metaphysis. This was also reported by Alberty, Peltonen and Ritsilä (1990). Alberty and Peltonen (1993) found some division of hypertrophic chondrocytes in the zone within two to three cells of the fracture separation layer, but no changes in the proliferative layer.

Our study has shown that the increased thickness of growth-plate cartilage produced by distraction in our model is not due to increased cell proliferation in the proliferative zone. On the contrary, cell division was reduced by distraction maintained for ten days. We found no evidence of anomalous cell proliferation in the hypertrophic or reserve zones. Normally, chondrocytes in the reserve zone divide very occasionally, and no cell proliferation takes place in the hypertrophic zone (Kember 1983).

Since fracture occurred at about four to five days and there was no significant increase in length in group-B animals, we conclude that the significant increase in tibial length in group C was due entirely to distraction of the fracture gap.

In our group A, the application of external fixation pins and a frame with no distraction caused no detectable abnormalities; the changes in the other two groups must have been caused mainly by the axial tension. The blood supply to chondrocytes in the proliferative zone is from loops of epiphyseal arteries, and the occasional necrosis and absence of cell proliferation in the proliferative zone may therefore be the result of epiphyseal ischaemia. Elmer et al (1992) found some histological damage within growth plates adjacent to the sites of epiphyseal pins, even where there was no encroachment upon the plate itself. In our studies the periosteum did not rupture and it seems possible that stretching of the periosteum, through which the blood supply must pass, may account for the ischaemia at both the metaphyseal and epiphyseal sides of the growth plate.

The apparent increase in cellularity of the growth plate associated with distraction at the given small levels of force was not due to increased cell proliferation; the thickening of the plate was probably caused by the delayed mineralisation of hypertrophic cells (although not directly shown) so that hypertrophic chondrocytes accumulated.

The complete absence of active dividing cells in some specimens, and disordered areas of necrosis in the proliferative zone in others, suggest that the distraction technique which we used in our experiment damaged the growth plate. We have not studied the ultimate influence on total growth, but extrapolation of the force levels which we used to the clinical situation shows that they are very close to, or lower than, the forces used for the most gradual distraction of the physis in young patients (Kenwright et al 1990), and therefore these treatments may cause similar abnormalities of growth-plate function. This supports statements by other authors, who have warned against the use of excessive force and against distraction in younger patients in whom loss of future growth from a distracted physis will lead to significant loss of leg length (Fishbane and Riley 1978; Monticelli and Spinelli 1981b; Canadell and de Pablos 1985).

Sledge and Noble (1976) used *bulk* counting of tritiated thymidine uptake to show that there was more uptake and therefore more dividing cells in the distracted growth plates. This method, however, does not identify those cells that are actually dividing; it is possible that other cells, such as those in the fracture zone and in invading blood vessels, were included in the bulk count. Elmer et al (1992) used tritiated thymidine autoradiography and showed in their animal model of chondrodiatasis that there was no change in cell proliferation in distracted growth plates. Our model differs from theirs in that it caused physeal fracture; this additional insult may adversely affect cell proliferation.

Our results suggest that physeal distraction does not stimulate cell proliferation in the growth plate, even when it is seen to be thickened after chrondrodiatasis. Previous workers may have misinterpreted the increase in thickness produced by physeal distraction as due to increased activity.

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