Effects of distraction and compression on proliferation of growth plate chondrocytes

A study in rabbits

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An external fixation device was applied across the distal femoral physis in 30 rabbits, and distraction or compression was performed for 3–21 days; either no operation or a sham-operation was performed on the contralateral side. Proliferating cells were labeled with 5-bromo-2′-deoxyuridine, a thymidine analogue, and subsequently localized in decalcified histologic sections using a specific monoclonal antibody.

The height of the proliferative and hypertrophic zones was increased after distraction; additionally, fracture-separation through the hypertrophic zone or at the junction of the physis and metaphysis was seen in 13 of the 15 specimens. Labeled cells were encountered only in the proliferative zone in all specimens except after early distraction, where labeled chondrocytes were seen close to the separation gap in the hypertrophic zone, too. Distraction had no effect on the number of labeled cells. A reduction in the height of the proliferative and hypertrophic zones occurred after compression, and the number of proliferating chondrocytes decreased.

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Transient hyperplasia of the physis is common during physeal distraction (Noble et al. 1978, Sledge and Noble 1978, De Bastiani et al. 1986, Elmer et al. 1989, Alberty et al. 1990, De Pablos and Canadell 1990). Fracture-separation of the growth plate occurs in rabbits, if the distraction force exceeds 2 kp (Noble et al. 1978, Sledge and Noble 1978, Nakamura et al. 1991). Moreover, in various animals, separation takes place if the daily rate exceeds 0.5 mm (De Bastiani et al. 1986, Fjeld and Steen 1988, De Pablos and Canadell 1990).

Compression of the growth plate is known to result in either a transient retardation or permanent arrest of growth (Haas 1945, 1948, Siffert 1956). It has been used to equalize leg-length inequality (Haas 1945, Blount and Clarke 1949). Few experimental studies have investigated how compression affects growth plate chondrocytes, however (Trueta and Trias 1961, Wilson-MacDonald et al. 1990).

High-resolution autoradiography using tritiated thymidine permits detection of cells that are in the process of DNA-synthesis (the S-phase of the cell cycle). A more rapid and non-radioactive immunohistochemical technique using 5-bromo-2′-deoxyuridine (BrdUrd), has recently been developed. BrdUrd, a thymidine analogue, is incorporated into newly synthesized DNA via the same pyrimidine pathway as thymidine, and localized using a specific monoclonal antibody (Gratzner 1982). BrdUrd labeling has been used for both undecalcified and decalcified bone and cartilage (Apte 1988, 1990, Apte and Puddle 1990, Farquharson and Loveridge 1990).

We used BrdUrd to label growth plate chondrocytes after physeal distraction or compression. The aim of this study was to determine whether mechanical forces had any stimulating or inhibiting effects on the proliferative activity of the growth plate and on growth plate morphology.

Material and methods

Operative procedure

30 New Zeeland white rabbits, 5–6 weeks of age, were operated on. Either distraction or compression of the left distal femoral physis was performed on 15 rabbits each. General anesthesia was accomplished with Hypnorm® (Janssen Pharmaceutica, Belgium). A circular external fixator (Fixel®, AMP Medical, France) was applied by drilling percutaneously 2 pairs of 1 mm Kirschner wires crosswise through the distal femoral epiphysis and the diaphysis. Distraction or compression was started on the day of operation. 15 rabbits received distraction at the rate of 0.7 mm once a day for 3–21 days. Another 15 received compression at the rate of 0.5 mm once a day up to 7 days, after

which the apparatus was left in situ for static compression for the rest of the time. The contralateral femur served as a control; either no intervention or a sham operation, where 2 fixation pins without the apparatus were inserted in the epiphysis, was performed. The animals were killed the following day after the distraction or compression was stopped, 1 hour after labeling with BrdUrd. Pin placement was controlled during preparation, and after preparation a micrometer was used to measure the length of both femurs.

Labeling and detection of proliferative cells

For cell labeling, 40 mg/kg of BrdUrd (Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in phosphate buffered saline, at a concentration of 5 mg/mL. The solution was injected into an ear vein, and the animals were killed 1 hour later by administering an overdose of pentobarbital. The distal femurs were dissected, cut into slices 3 mm thick in the coronal plane (Apte 1988) and fixed for 24 hours in Bouin's fluid (a solution consisting of 75 mL of picric acid, 25 mL of 37.4% formaldehyde, and 5 mL of glacial acetic acid, which was added immediately before use). The slices were then washed with 80% (v/v) ethanol, decalcified for 10–14 days in several changes of 10% (v/v) formic acid at 4 °C, dehydrated in a series of alcohol, embedded in paraffin, and cut into sections 6–7 μ thick. For cell counting and measurement of the physeal zones the sections were cut from the middle slice parallel to the longitudinal cell columns.

A cell proliferation kit (Amersham International, Amersham, UK) was used for detection of labeled cells. To allow antibody access, denaturation of cellular DNA was accomplished by nuclease digestion simultaneously with antibody incubation (Gonchoroff et al. 1986). Detection of the bound antibody was achieved using peroxidase conjugated antibody to mouse immunoglobulin polymerizing diaminobenzidine in the presence of cobalt and nickel, which gives blue-black staining at sites of BrdUrd incorporation. The slides were counterstained with Mayer's hematoxylin and viewed under a light microscope.

Measuring the physeal zones

Measurement of the height of the physeal zones was performed using the semiautomatic Videoplan Image Processing System (Kontron Elektronik Co, Eching, Germany) connected via a television camera to a Leitz Diaplan light microscope (Ernst Leitz Wetzlar Co., Wezlag, Germany). A magnification of 10× or 25× was used on the microscope, the magnification of the computer itself being 1× or 0.63×, respectively. The microscopic field was displayed on the TV screen of the computer and the measurements were performed by using a digitizer tablet and a cursor linked to it. The height of each physeal zone was measured 3 times in 3 distinct locations of 1 section (Figure 1). The mean of these 9 measurements was determined to represent the height of the physeal zone in that particular specimen.

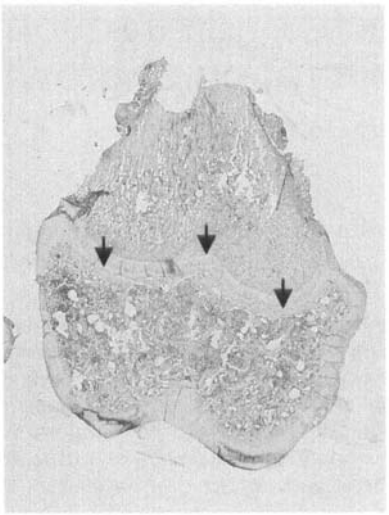


Figure 1. The arrowheads indicate the 3 measurement sites of the physeal zones (control specimen). 3 measurements of each zone were made at each site. Mayer's Hematoxylin, ×3.0

Counting proliferating chondrocytes

Counting of the BrdUrd labeled cells was performed using an eyepiece graticule (Miller square with a ruled area of 7 × 7 mm). A magnification of 25× was used, allowing the whole height of the reserve zone and proliferative zone to be included in 1 area. The whole width of the physis was studied. 5 or more countings were made from 1 section, and the average number of labeled cells per area was calculated. The lowest and highest values were excluded and the mean of the remaining values was used for statistical analysis.

Statistics

To estimate the accuracy of both the zone height and cell counting measurements, 30 consecutive measurements were made of the distance between 2 fixed points using the Videoplan Image Processing System, and, correspondingly, labeled cells of 1 particular section were counted 30 times.

The Mann-Whitney U-test for unpaired data was used to compare the sham-operated and non-operated controls. The Wilcoxon signed rank test for paired data was used to compare the distracted and com-

pressed specimens with the corresponding control. No distinction was made between the non-operated and sham-operated controls.

Results

The coefficient of variation of the 30 distance measurements was 2.2 percent, and of the 30 cell countings 10 percent. Comparison of the sham-operated and non-operated control physes (n 30) revealed no difference between the number of labeled proliferative chondrocytes or between the heights of the physeal zones.

There were no serious complications leading to premature removal of the device in this series.

Effects of distraction

The average daily increase in length of the distracted femur was 0.4 (0.2–0.7) mm (Table 1). Separation through the hypertrophic zone or at the junction of the hypertrophic zone and the metaphysis occurred in 13/15 specimens.

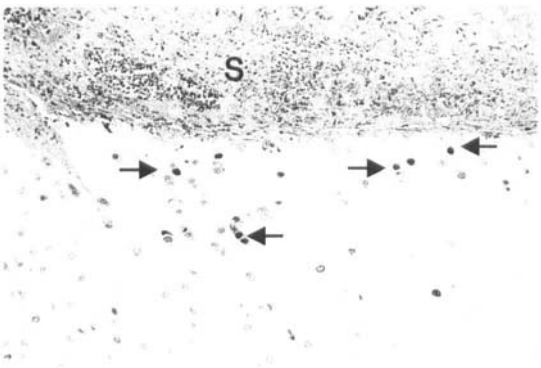


Figure 2. Labeled cells (arrows) in the hypertrophic zone adjacent to the separation gap in a specimen distracted for 3 days. S separation gap. Mayer's Hematoxylin, ×102.

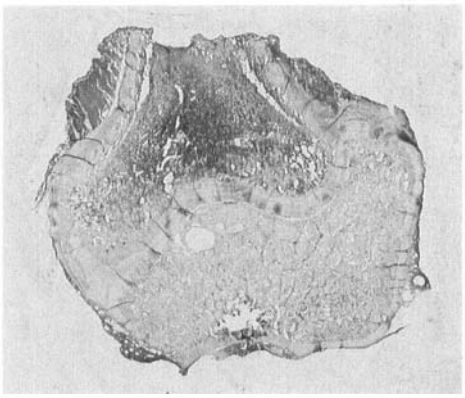
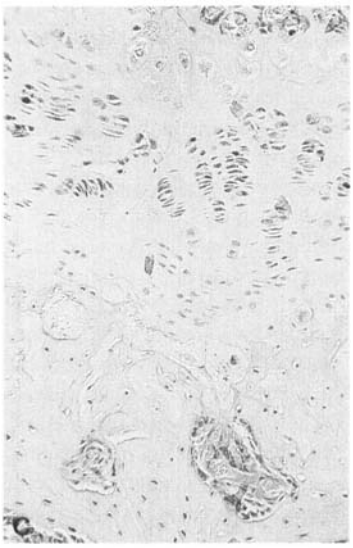


Figure 4. Specimen after 14 days of compression. The epiphysis and physis are bulging over the edges of the metaphysis. Mayer's Hematoxylin, ×3.0.

Figure 3. The appearance of the physis.

Control physis. The labeled cells are stained dark. Mayer's Hematoxylin, ×116.

The physis after 3 days of distraction. Increased height of both the proliferating zone and hypertrophic zone. The separation gap at the top is marked with an arrow. Mayer's Hematoxylin, ×40.



Specimen after 14 days of compression. Diminished height of the physis, distorted short columns, and very few labeled cells. Mayer's Hematoxylin, ×116.

Table 1. Data on the rabbits subjected to distraction

A	B	C	D	E	F	G	H	I	J	K	L	M
D1	1	3	N	0.5	12	13	.11	.12	.37	.56	.31	1.2
	2	3	N	1.5	8.3	13	.06	.08	.15	.48	.16	.51
	3	3	N	2.0	6.1	11	.06	.10	.15	.54	.22	.65
	4	5	S	1.8	9.9	2.8	.05	.06	.17	.54	.15	.54
	5	5	N	2.0	7.0	6.8	.06	.06	.23	.34	.28	1.1
	6	5	N	1.2	6.5	8.0	.06	.05	.17	.28	.19	1.0
	7	7	S	4.0	8.9	7.2	.05	.05	.16	.23	.22	.77
	8	7	N	3.0	9.0	5.1	.05	.06	.15	.25	.19	.83
	9	7	S	4.0	7.5	6.9	.04	.05	.14	.24	.19	1.1
D2	10	14	S	5.0	7.1	7.5	.05	.07	.15	.19	.23	.43
	11	14	S	5.0	12	6.3	.05	.05	.13	.13	.28	.63
	12	14	S	5.0	9.4	8.3	.06	.06	.16	.14	.30	.27
	13	21	N	8.5	13	1.0	.09	.06	.25	.10	.31	.19
	14	21	N	7.5	6.8	6.6	.10	.12	.20	.50	.32	1.5
	15	21	S	9.5	3.1	4.4	.07	.13	.15	.27	.19	1.2

A Group
In Group D1 distraction was performed for 3, 5 or 7 days and in Group D2 for 14 or 21 days. The rate was 0.7 mm once a day in both groups.
B Rabbit
C Time of distraction in days
D Control physis
N Non-operated
S Sham-operated
E The increase in length (mm) of the distracted femur compared with the contralateral side
F Number of labeled chondrocytes in control physes
G Number of labeled chondrocytes per area in distracted physes
H Reserve zone height of control physes (mm)
I Reserve zone height of distracted physes (mm)
J Proliferative zone height of control physes (mm)
K Proliferative zone height of distracted physes (mm)
L Hypertrophic zone height of control physes (mm)
M Hypertrophic zone height of distracted physes (mm)

Comparison of the distracted and control specimens revealed no change in the number of BrdUrd-labeled cells in the physes. Labeled chondrocytes were seen almost exclusively in the proliferative zone and only occasionally in the reserve zone among all controls and among group D2 (distraction of 14–21 days). 8 specimens belonging to group D1 (distraction of 3–7 days), however, had BrdUrd-labeled chondrocytes in the lower hypertrophic zone. The labeled cells were located within a distance of 2–3 cells from the separation gap (Figure 2). The total number of labeled hypertrophic cells in one section varied from 5–20.

Both hyperplasia of the physis and columnar disorganization were detected (Figure 3B). The height of the proliferative and hypertrophic zones was significantly increased in all distracted specimens (groups D1 and D2 analyzed together, P 0.007 for proliferative and P 0.002 for hypertrophic). When groups D1 and D2 were analyzed separately, the increase in height of the proliferative and hypertrophic zones was significant only in the former (P 0.008 for both). A nearly significant increase in the reserve zone height was detected only in group D1 (P 0.05).

Effects of compression

The average daily shortening of the compressed femurs was 0.3 (0–0.4) mm (Table 2). The metaphyseal area of the distal femur appeared to be funnel-shaped and the physis seemed to be bulging over the edges of the metaphysis (Figure 4). The number of labeled chondrocytes in the compressed physes was lower than in controls (Groups C1 and C2 analyzed together, $P < 0.001$). The chondrocyte columns were distorted and crooked. The hypertrophic chondrocytes were degenerated, and the basal hypertrophic chondrocytes occurred occasionally as empty shells (Figure 3C). The decrease in the height of the proliferative and hypertrophic zones was significant ($P < 0.001$ for proliferative and P 0.003 for hypertrophic), but no change in reserve zone height was detected. After compression for 21 days, the physis was markedly narrowed but still identifiable; and a small bone bridge was seen in only one specimen.

Table 2. Data on the rabbits subjected to compression

A	B	C	D	E	F	G	H	I	J	K	L	M
C1	16	3	N	–1.0	6.4	2.2	.08	.06	.18	.17	.30	.42
	17	3	N	–0.5	8.2	1.6	.09	.06	.23	.17	.34	.18
	18	3	S	–0.5	13	0.8	.08	.06	.23	.17	.33	.20
	19	5	S	0.0	5.5	2.1	.07	.07	.18	.11	.36	.17
	20	5	S	–0.5	7.4	2.6	.07	.07	.25	.10	.39	.17
	21	5	N	–1.0	11	1.9	.06	.06	.19	.11	.34	.21
	22	7	S	–2.7	11	2.1	.07	.07	.21	.08	.34	.14
	23	7	S	–2.8	9.9	0.3	.06	.07	.23	.07	.33	.23
	24	7	N	–2.8	7.9	0.4	.07	.07	.20	.10	.33	.19
C2[a]	25	14	S	–4.0	5.5	1.3	.05	.06	.17	.05	.23	.17
	26	14	S	–4.0	8.0	1.3	.05	.07	.18	.05	.33	.12
	27	14	N	–5.5	17	2.3	.07	.06	.27	.14	.38	.25
	28	21	N	–7.0	5.6	0.8	.06	.10	.17	.10	.28	.16
	29	21	N	–6.5	5.3	1.5	.07	.07	.15	.11	.28	.28
	30	21	S	–6.0	6.3	1.7	.06	.08	.18	.07	.28	.21

A Group
In Group C1 compression was performed for 3, 5 or 7 days and in Group C2 for 14 or 21 days.
[a]The rate was 0.5 mm once daily up to 7 days, after which the apparatus was locked for static fixation
B Rabbit
C Time of compression in days
D Control physis
N Non-operated
S Sham-operated
E The decrease (–) in length (mm) of the compressed femur compared with the contralateral side
F Number of labeled chondrocytes per area in control physes
G Number of labeled chondrocytes per area in compressed physes
H Reserve zone height of control physes (mm)
I Reserve zone height of compressed physes (mm)
J Proliferative zone height of control physes (mm)
K Proliferative zone height of compressed physes (mm)
L Hypertrophic zone height of control physes (mm)
M Hypertrophic zone height of compressed physes (mm)

Discussion

Apte (1990) has assessed the validity of BrdUrd immunohistochemistry for localization of S-phase cells in decalcified tissues. Double labeling of the proximal tibial growth plate chondrocytes with tritiated thymidine (3H-TdR) and Brd yielded a highly significant correlation between the distribution of BrdUrd labeled cells and the sites of cell proliferation determined with thymidine.

In our study use of an eyepiece graticule proved to be the best method for counting labeled cells in a specific area. Columnar distortion in distracted and compressed specimens made it impracticable to count labeled cells in a vertical column. Measuring the labeling index (Kember 1971) was not reliable either, as the number of proliferating cells in each field varied greatly, especially between compressed and control specimens.

Cell proliferation in the physis occurs principally in the proliferative zone and only sporadically in the reserve zone (Kember 1960, Kember and Walker 1971, Apte 1988, 1990, Farquharson and Loveridge 1990). The basal hypertrophic chondrocytes do not proliferate, even though they are known to be metabolically active (Farnum et al. 1990). In studies where cell proliferation has been measured in connection with distraction, proliferating chondrocytes have not been reported outside the proliferative zone (Noble et al. 1978, Elmer et al. 1989, Kenwright et al. 1990). In the present study, proliferative activity also occurred in some hypertrophic chondrocytes after 3–7 days of distraction. It is known that phenotypic expression of chondrocytes is regulated by different sets of hormones and growth factors (Kato and Gospodarowicz 1985, Iwamoto et al. 1989), the basal hypertrophic cell representing the last developmental stage of the proliferative zone chondrocyte. Separation and hematoma formation in the basal hypertrophic zone may inhibit the normal chondrocyte transformation sequence. Our results suggest that hypertrophic chondrocytes have the ability to proliferate in circumstances where normal maturation is affected.

The long-term effects of distraction on the physis are still not fully known, but premature closure of the distracted physis and/or loss of achieved lengthening have often been reported (Fjeld and Steen 1988, 1990, De Pablos and Canadell 1990). The biologic mecha-

nism of physeal hyperplasia after distraction is not clear, and opinions differ as to whether leg lengthening can be produced by mechanical stimulation of growth and not merely by producing a fracture through the plate.

Physeal distraction leads to hyperplasia of the proliferative and hypertrophic zones (Noble et al. 1978, Sledge and Noble 1978, Connolly et al. 1986, Alberty et al. 1990, Kenwright et al. 1990). This hyperplasia occurs whether or not physeal separation occurs (Sledge and Noble 1978, Alberty et al. 1990). In the present study, the increase in proliferative and hypertrophic zone heights was most marked during the first week of distraction, whereafter a return towards normal zone height was seen.

The means by which chondrocytes modulate longitudinal bone growth are not fully known. Changes in the size of the pool of proliferating cells and in the proliferation rate (Kember and Walker 1971, Kember 1978), height (Hunziker and Schenk 1989) and volume (Breur et al. 1991) of the terminal hypertrophic cell have been implicated. Seinsheimer and Sledge (1981) found that there was a significant correlation between growth rate and growth plate thickness, rate of division of proliferative chondrocytes and [^{35}S] sulphate incorporation.

Reports on cell proliferation after physeal distraction are few. Sledge and Noble (1978) performed scintillation-counting of growth plates harvested in toto, observing increased thymidine uptake in both non-separated physes and in physes with microfractures. Noble et al. (1978) reported increased cell proliferation on the distracted side, and Kenwright et al. (1990) stated that the increased cell proliferation occurred in the proliferative zone of non-separated physes. Elevated protein content and synthesis of sulfated polysaccharides of the growth plate after distraction have also been reported (Noble et al. 1978, Sledge and Noble 1978). Elmer et al. (1989), however, saw no increase in the percentage of labeled cells in the physis, or in the number of cells per columns in spite of widening of the proliferative zone and/or the hypertrophic zone. It is noteworthy that increased proliferation or metabolism have been observed after distraction with spring loaded devices only, in physes with no separation (Kenwright et al. 1990) or at the most microfractures (Noble et al. 1978, Sledge and Noble 1978). Hert (1969) reported radiographically measurable stimulation of physeal growth and thickening of the physis after force-regulated distraction with a spring-loaded device. A spring-loaded device may ensure a more even increase of the tensile forces across the physis than a turnbucle device, and this could play a role in activation of cell proliferation. Nakamura et al. (1991) found no evidence of growth stimulation when continuously monitoring the forces during gradual physeal distraction. They concluded that distraction resulted in lengthening only if separation occurred, and that the lengthening effect was solely due to the fracture of the physis. In the present study, a clear increase in the proliferative zone height was seen despite separation; nevertheless, the height increase was not associated with an increase of proliferation either during early distraction or later. We cannot totally exclude an acceleration of physeal growth, however, because changes in hypertrophic cell volume and/or height seem to be the best indicators of growth rate changes, according to the latest reports (Hunziker and Schenk 1989, Breur et al. 1991). Acceleration of the growth rate has even been found to be associated with a decrease in growth plate height (Hunziker and Schenk 1989). Moreover, we do not know whether the synthesis phase and cell cycle time is affected by local mechanical forces. Slight changes in the rate of cell proliferation probably would not be detectable if a labeling time of one hour were used.

Compression of the physis affects both the morphology and function of the chondrocytes (Siffert 1956, Trueta and Trias 1961, Christensen 1973, Wilson-MacDonald et al. 1990), and compression by forces well in excess of body weight will eventually cause physeodesis (Trueta and Trias 1961). Farquharson and Loveridge (1990) found that the labeling index in the growth plate decreased with advancing age in rats, and Hunziker and Schenk (1989) found a simultaneous decrease of cell height, cell volume and proliferation rate in the longitudinal direction in the physis in association with physiological reduction of the growth rate.

In our study, the force used for compression was progressive during the first week, and thus markedly beyond physiological loading. We did not see thickening of the physis as described by Trueta and Trias (1961). On the contrary, a reduction in proliferative and hypertrophic zone heights was evident. Cell proliferation in the proliferative zone was almost arrested. In spite of the marked disturbance of columnar architecture and narrowing observed after 21 days of compression, bone bridges were seen in only one case, implying the possibility of restoration of physeal growth if the compression were discontinued.

We conclude that gradual physeal distraction results in an increase in proliferative and hypertrophic zone heights, but does not increase chondrocyte proliferation, as measured by labeling proliferating cells in the S-phase. The effect of distraction on physeal growth regulation mechanisms remains uncertain. Compression, on the other hand, leads to a significant reduction in proliferative and hypertrophic zone heights and diminished chondrocyte proliferation.

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