The structural architecture of adult mammalian articular cartilage evolves by a synchronized process of tissue resorption and neoformation during postnatal development

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Summary

Objective: During postnatal development, mammalian articular cartilage acts as a surface growth plate for the underlying epiphyseal bone. Concomitantly, it undergoes a fundamental process of structural reorganization from an immature isotropic to a mature (adult) anisotropic architecture. However, the mechanism underlying this structural transformation is unknown. It could involve either an internal remodelling process, or complete resorption followed by tissue neoformation. The aim of this study was to establish which of these two alternative tissue reorganization mechanisms is physiologically operative. We also wished to pinpoint the articular cartilage source of the stem cells for clonal expansion and the zonal location of the chondrocyte pool with high proliferative activity.

Methods: The New Zealand white rabbit served as our animal model. The analysis was confined to the high-weight-bearing (central) areas of the medial and lateral femoral condyles. After birth, the articular cartilage layer was evaluated morphologically at monthly intervals from the first to the eighth postnatal month, when this species attains skeletal maturity. The overall height of the articular cartilage layer at each juncture was measured. The growth performance of the articular cartilage layer was assessed by calcein labelling, which permitted an estimation of the daily growth rate of the epiphyseal bone and its monthly length-gain. The slowly proliferating stem-cell pool was identified immunohistochemically (after labelling with bromodeoxyuridine), and the rapidly proliferating chondrocyte population by autoradiography (after labelling with $^{3}$H-thymidine).

Results: The growth activity of the articular cartilage layer was highest 1 month after birth. It declined precipitously between the first and third months, and ceased between the third and fourth months, when the animal enters puberty. The structural maturation of the articular cartilage layer followed a corresponding temporal trend. During the first 3 months, when the articular cartilage layer is undergoing structural reorganization, the net length-gain in the epiphyseal bone exceeded the height of the articular cartilage layer. This finding indicates that the postnatal reorganization of articular cartilage from an immature isotropic to a mature anisotropic structure is not achieved by a process of internal remodelling, but by the resorption and neoformation of all zones except the most superficial (stem-cell) one. The superficial zone was found to consist of slowly dividing stem cells with bidirectional mitotic activity. In the horizontal direction, this zone furnishes new stem cells that replenish the pool and effect a lateral expansion of the articular cartilage layer. In the vertical direction, the superficial zone supplies the rapidly dividing, transit-amplifying daughter-cell pool that feeds the transitional and upper radial zones during the postnatal growth phase of the articular cartilage layer.

Conclusions: During postnatal development, mammalian articular cartilage fulfils a dual function, viz., it acts not only as an articulating layer but also as a surface growth plate. In the lapine model, this growth activity ceases at puberty (3–4 months of age), whereas that of the true (metaphyseal) growth plate continues until the time of skeletal maturity (8 months). Hence, the two structures are regulated independently. The structural maturation of the articular cartilage layer coincides temporally with the cessation of its growth activity — for the radial expansion and remodelling of the epiphyseal bone — and with sexual maturation. That articular cartilage is physiologically reorganized by a process of tissue resorption and neoformation, rather than by one of internal remodelling, has important implications for the functional engineering and repair of articular cartilage tissue.

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Introduction

The bony ends of mammalian synovial joints are lined with a layer of articular cartilage, which permits congruency between the two opposing skeletal elements, facilitates the transfer of load between these, their practically frictionless movement, and acts as a stress absorber during sustained static loading$^{1,2}$.

During postnatal development, the long bones undergo extensive growth and remodelling in all regions, but particularly in the epiphysis. In the epiphysis, these activities are governed by the articular cartilage layer, which thus functions also as a surface growth plate for the epiphyseal bone during the postnatal period [Fig. 1(A)]. Growth of the metaphysis and the diaphysis is governed by the “true” growth plate, viz., by the physis$^{3}$.

Mature articular cartilage is characterized by a high degree of structural anisotropy, its cells being organized into well-defined vertical columns and horizontal strata$^{4,5}$. However,
ferred to as an “articular epiphyseal complex”9,10. Although immature mammalian articular cartilage is sometimes re-growth and remodelling of the adjacent bony ends9. Hence, the immature tissue acts not only as an articulating layer, but also as an epiphyseal plate, whose activities govern the postnatal growth activities and structural reorganization. Later, they hypertrophy and initiate matrix mineralization. Longitudinal bone growth is achieved both by rapid clonal expansion in the vertical direction and by cell hypertrophy (modified from Lavker and Sun, 2000)27.

Materials and Methods

ANIMALS

A total of 38 New Zealand white rabbits were used for this study. In this species, puberty occurs 2–3 months after birth and corresponds to sexual maturity. Skeletal maturity (closure of all physes) is attained 8 months after birth. Our analysis was confined to the high-weight-bearing (central) areas of the medial and lateral femoral condyles. Following birth, 4–5 rabbits were sacrificed at monthly intervals up to 8 months. Three animals were sacrificed at birth. The rabbits were killed by administering an overdose of Pentothal and Narcan. Various parameters of growth performance were quantified at each monthly interval. These included the daily growth rate of the epiphyseal bone (determined after labelling with calcein)3 and its monthly length-gain (see below).

DESCRIPTIVE AND MORPHOMETRIC PARAMETERS

At each time-point, the structural organization of the articular cartilage layer was evaluated in descriptive terms on the basis of observations in the light and electron microscopes. The degree of cell anisotropy was quantified by determining the anisotropy coefficient (see below). The overall height of the articular cartilage layer was evaluated in descriptive terms on the basis of observations in the light and electron microscopes. The degree of cell anisotropy was quantified by determining the anisotropy coefficient (see below). The overall lengthening of the epiphyseal bone were analysed on a monthly basis from the time of birth to that of skeletal maturity. By comparing the latter data with the corresponding monthly changes in the overall height of the articular cartilage layer, we were able to gain an insight into the mechanism underlying the structural reorganization of this tissue during its maturation. The location of the slowly proliferating stem-cell pool was identified immunohistochemically (after long-term labelling with bromodeoxyuridine) and that of the rapidly amplifying daughter-cell pool by autoradiography (after short-term labelling with 3H-thymidine)12.

Fig. 1. (A) Scheme representing postnatal growth of the epiphysis (E) of a long bone. The articular cartilage (A, yellow) acts not only as an articulating layer, but also as a surface growth plate for the longitudinal, radial and lateral growth of the epiphyseal bone (radially oriented arrows). The true growth plate (G, green), which is located between the epiphysis (E) and the metaphysis (M), is responsible for the longitudinal growth of the metaphysis (M) and the diaphysis (D). (B) Scheme illustrating the bidirectional replication of superficial-zone stem cells during the growth-activity phase of the articular cartilage layer. This slowly proliferating pool supplies daughter cells which can be displaced either horizontally (1) or vertically (2). Horizontally displaced cells replenish the stem-cell pool and effect lateral growth of the articular cartilage layer. Vertically displaced cells feed the rapidly proliferating pool of transit-amplifying cells in the transitional and upper radial zones. These latter cells effect rapid clonal expansion in the vertical direction. Later, they hypertrophy and initiate matrix mineralization. Longitudinal bone growth is achieved both by rapid clonal expansion in the vertical direction and by cell hypertrophy (modified from Lavker and Sun, 2000)27.

at birth and during postnatal growth, the articular cartilage layer is more isotropic in structure4,6 and bears little resemblance to that of the adult organism7,8. As aforementioned, the immature tissue acts not only as an articulating layer but also as an epiphyseal plate, whose activities govern the growth and remodelling of the adjacent bony ends9. Hence, immature mammalian articular cartilage is sometimes referred to as an “articular epiphyseal complex”9,10. Although the dual functionality of immature mammalian articular cartilage is generally recognized, the mechanisms underlying its postnatal growth activities and structural reorganization remain obscure2,11. A clarification of this issue would not only further our understanding of the physiological process per se, but also guide our endeavours to engineer articular cartilage tissue in the context of repair.

We hypothesize that the physiological reorganization of the articular cartilage layer within synovial joints is based either on a process of internal tissue remodelling, or on one of tissue resorption (i.e., elimination by controlled vascular invasion from the underlying bony compartment) synchronized with appositional growth from the superficial zone.

It was the purpose of the present study to elucidate which of these two mechanisms is operative in the knee-joint cartilage of New Zealand white rabbits. The growth performance of the articular cartilage layer and the associated lengthening of the epiphyseal bone were analysed on a monthly basis from the time of birth to that of skeletal maturity. By comparing the latter data with the corresponding monthly changes in the overall height of the articular cartilage layer, we were able to gain an insight into the mechanism underlying the structural reorganization of this tissue during its maturation. The location of the slowly proliferating stem-cell pool was identified immunohistochemically (after long-term labelling with bromodeoxyuridine) and that of the rapidly amplifying daughter-cell pool by autoradiography (after short-term labelling with 3H-thymidine)12.

SECONDARY DEFINITION OF THE ZONES COMPRISING THE ARTICULAR CARTILAGE LAYER

The zonal organization of the articular cartilage layer (Fig. 2) was defined quantitatively as previously described9,12. The height of the superficial (tangential) zone was defined on the basis of cell morphology and...
confirmed autoradiographically [absence of a signal following short-term labelling with $^{3}$H-thymidine (see below)] to be 1/13th of the overall height of the cartilage layer, from the surface down to the cartilage—bone interface [i.e., the resorption (vascular invasion) front in immature articular cartilage]. The transitional zone was defined as 2/13ths, and the radial zone as 10/13ths of the overall height. The upper and lower portions of the radial zone were divided into equal parts (each constituting 5/13ths of the overall height of the articular cartilage layer). During the early postnatal phase (0–3 months), the calcified cartilage zone is defined as the region in which the longitudinal septa are mineralized. It extends from the mineralization front down to the vascular invasion front. During the late postnatal phase (3–8 months), the calcified cartilage zone is defined as the region in which both the longitudinal and the horizontal septa are mineralized. It extends from the mineralization front (i.e., the tidemark) down to the upper boundary of the subchondral bone plate.

**Determination of the Daily Growth Rate of the Epiphyseal Bone by Labelling with Calcein**

The daily growth rate of the epiphyseal bone lying just beneath the articular cartilage layer was determined on the basis of the tetracycline-labelling principle. Rabbits were subcutaneously injected with a single dose of calcein (15 mg/kg of body weight) either 3 days (in young animals with a high growth rate) or 8 days (in older animals with a lower growth rate) prior to sacrifice. In younger animals with a high growth rate, a longer time interval is necessary to catch the labelled front before it advances too far into the epiphyseal bone trabeculae. In older animals with a lower growth rate, a longer time interval is necessary to yield a labelled front of measurable distance.

**Identification of the Slowly and Rapidly Proliferating Cell Pools**

**A. Labelling of the Slowly Proliferating (Stem-Cell) Pool with Bromodeoxyuridine**

Labelling with bromodeoxyuridine (an analogue of thymidine) was used to identify the pool of cells with a very slow rate of proliferation, viz., the stem-cell population. Since bromodeoxyuridine has a half-life (of a few hours’ duration in the synovial fluid as well as in the serum), and in order to avoid false negative results generated by possible diurnal variations in cell-proliferation rates, it must be administered continuously on a daily basis for a period that covers the estimated cycling time of the stem cells, viz., 12 days. It was added to the rabbits’ daily supply of sterile drinking water during the last 12 days preceding sacrifice.

**B. Labelling of the Rapidly Proliferating Daughter-Cell Pool with $^{3}$H-Thymidine**

Labelling with $^{3}$H-thymidine was used to identify the pool of cells undergoing rapid proliferation. $^{3}$H-thymidine (total dose: 400 mCi in 1- and 2-month-old rabbits; 700 mCi in 3- and 8-month-old rabbits) was injected intra-articularly into each knee joint 1 day and 2 days prior to sacrifice. Since $^{3}$H-thymidine has a half-life in the synovial fluid of only 30–40 min to maximally a few hours, it will label (and with high sensitivity) only very rapidly proliferating cell pools. The rabbits were housed in special pharmacological cages from which the radioactive excreta were collected and safely disposed of. $^{3}$H-thymidine was detected immunohistochemically according to the method described by Wilmsen et al. (see below).

**Histology**

Tissue blocks obtained from the high-weight-bearing (central) area of each medial and lateral femoral condyle were processed for light microscopy, immunohistochemistry and autoradiography.

For light microscopy, the tissue was chemically fixed in 2% glutaraldehyde solution buffered with 0.1 M sodium cacodylate (pH 7.4, 330 mOsm). Ruthenium hexaamine trichloride (RHT) (0.7%) was added to this medium 10 min
described. Six-hundred-micrometre-thick slices were pre-
hanol and embedded in methylmethacrylate, as previously
buffered with 0.1 M sodium cacodylate (pH 7.4) for 2 days
dyles were chemically fixed in 4% formaldehyde solution
rabbits, tissue blocks derived from the distal femoral con-
sections were prepared for examination in a Hitachi
Eclipse, light microscope. These sections were stained
for observation in an Olympus Vanox AH 2, or a Nikon
Eclipse, light microscope. These sections were stained
for light microscopy at low magnifications.
For incident-light fluorescence microscopy of calcein-la-
telled material (in an Olympus Vanox AH2, or a Nikon
Eclipse, light microscope), selected tissue blocks were fixed in
70% ethanol to avoid the autofluorescence effect of glu-
dimaldehyde. They were then dehydrated in ethanol and em-
bedded in methylmethacrylate. Vertical sections, 12
micrometre-thick sections were prepared using a Leica ultrami-
crotome E. One-micrometre-thick sections were prepared for
observation in a Hitachi 7100 B electron microscope. In newborn
and 1-month-old rabbits, tissue blocks derived from the distal femoral con-
dyles were chemically fixed in 4% formaldehyde solution
buffered with 0.1 M sodium cacodylate (pH 7.4) for 2 days at ambient

temperature. After rinsing in isotonic sodium cacodylate ca-
codylate buffer (pH 7.4), the material was dehydrated in ethan-
ol and embedded in methylmethacrylate, as previously
described20 Six-hundred-micrometre-thick slices were pre-
filled with McNeal’s Tetrachrome and basic
fuchsin (according to Schenk et al21) in preparation for
light microscopy at low magnifications.
For the immunohistochemical analysis of material la-
talled with bromodeoxyuridine, tissue blocks were chemi-
cally fixed in 4% formaldehyde solution buffered with
0.1 M sodium cacodylate (pH 7.4) for 2 days at ambient

temperature. They were then decalcified in 2% ethylenedi-
amine tetra-acetic acid solution for 2–3 weeks, likewise at
ambient temperature. Thereafter, they were dehydrated in
ethanol and embedded in paraffin. Sections, 5–7 µm in

thickness, were prepared using a Jung rotatory microtome.
The bromodeoxyuridine-labelled epitopes were identified
on deparaffinized sections using a commercial antibody
(Sigma AG, Buchs, Switzerland).
Tissue blocks destined for 3H-thymidine autoradiography
were chemically fixed in 2% glutaraldehyde solution con-
taining 0.5% cetylpyridinium chloride instead of RHT (which
absorbs radiation). Thereafter, the tissue was processed for
embedding in epoxy resin as described above. One-micro-
metre-thick sections were prepared using a Leica ultrami-
crotome E. These were covered with Kodak 827 emulsion
in the dark and exposed for 6 days prior to development12.

ESTIMATION OF THE HEIGHT OF THE SUPERFICIAL
(STEM-CELL) ZONE
In the 3H-thymidine-labelled tissue sections, only the rap-
idly proliferating cell pools are tagged and revealed by au-
roradiography. These cells were located in the transitional
and upper radial zones (see Results). By measuring the dis-
tance between the articular cartilage surface and the upper
border of the transitional zone on autoradiographs, we were
able to estimate the height of the superficial (stem-cell)
zone. The measurements correlated very well with the
morphologically based determination of this zone’s height
(see above).

ESTIMATION OF THE HEIGHT OF THE ARTICULAR
CARTILAGE LAYER
The overall height of the articular cartilage layer, from the
surface down to the chondro-osseous junction, was mea-
sured in three adjacent tissue blocks derived from each
area (medial and lateral femoral condyles) using a parallel
line grid, as previously described6.

ESTIMATION OF THE DAILY GROWTH RATE OF THE
EPiphyseal BONE
Unstained, 7-µm-thick sections, prepared from tissue that
had been fixed in ethanol and embedded in methylmethacry-
late, were used to estimate the daily longitudinal growth rate
of the epiphyseal bone. The distance between the calcine-
labelled front and the vascular invasion front (i.e., the chon-
dro-osseous junction) was measured perpendicular to the
articular surface using a test system that consisted of parallel
longitudinal lines8. Two tissue blocks per condyle were used
for this purpose. Measurements were made at 3–4 equidis-
tant locations along the parallel longitudinal lines of the test
system, with a random start at the left-hand margin of the
section22. The daily longitudinal growth rate of the epiphyseal
bone was estimated by dividing the measured distance by the
number of labelling days (3 or 8)1322.

ESTIMATION OF THE MONTHLY LENGTH-GAIN
IN THE EPiphyseal BONE
The absolute monthly lengthening of the epiphyseal bone, viz.,
the monthly growth performance of the articular cartilage
layer, was determined by multiplying the daily
growth rate by the number of days in a month. This value
was then compared with the overall height of the articular
cartilage layer in order to elucidate the mechanisms under-
lying epiphyseal bone growth and articular cartilage
remodelling.

ESTIMATION OF THE DEGREE OF STRUCTURAL ANISOTROPY
WITHIN THE ARTICULAR CARTILAGE LAYER
The degree of structural anisotropy characterizing the ar-
ticular cartilage layer was described morphologically on the
basis of observations in the light and electron microscopes.
But in order to obtain a quantitative estimate of this param-
eter at the cellular level, an anisotropy coefficient was de-
dined and determined. This was obtained by dividing the
horizontal by the vertical diameter of a cell in centrally sec-
tioned profiles (i.e., those containing a nucleus). Anisotropy
coefficients were determined for all centrally sectioned cell
profiles in each zone and at each time-point. The measure-
ments were made on photographic prints at a final magnifi-
cation of 750 x.

STATISTICS
Statistical comparisons between the different age groups
were achieved using the unpaired t-test with a one-sided al-
ternative hypothesis23. The p-values were cross-checked
by the Wilcoxon-Rank-sum test, likewise using a one-sided
alternative hypothesis. At each time-point, the number of rab-
bits analysed (n-value) was 4–5; for each of these animals,
4–5 tissue blocks were evaluated. For the statistical descriptions and comparisons, Microsoft Excel software was used. Graphs were produced using Windows graphic software. Generally, the data are presented as mean values together with the standard error of the mean (S.E.M.).

Results

GENERAL MORPHOLOGICAL DESCRIPTION OF THE ARTICULAR CARTILAGE LAYER DURING POSTNATAL DEVELOPMENT

Morphological inspection of the articular cartilage layer revealed the tissue to undergo a fundamental process of reorganization during its postnatal development (Figs. 2 and 3).

ONE MONTH AFTER BIRTH

One month after birth, the superficial zone flat cells whose long axis is oriented parallel to the articular cartilage surface [Fig. 3(A)]. But in the underlying zones, the cells are arranged more isotropically. They exist singly or as small clusters with no preferential spatial orientation. However, a vectoral gradient in cell size and shape is apparent. In the transitional zone and in the upper and middle radial zones, the chondrocytes tend to be more rounded in shape than in the superficial zone, but they are still small in size. In the lower radial zone, they are larger (hypertrophic). In this latter region, only the longitudinal septa are mineralized; these are destined to become the future primary epiphyseal bone trabeculae. At this 1-month stage, the articular cartilage layer represents a relatively poorly organized surface growth plate. The high numerical density of blood vessels invading this layer at the chondro-osseous junction [Fig. 4(A)] is indicative of intensive tissue resorption and neoformation.

TWO MONTHS AFTER BIRTH

Two months after birth, the structural organization of the articular cartilage layer has not changed dramatically.
However, the cells are more anisotropically arranged and their numerical density has decreased. Moreover, individual cells are spatially more oriented, particularly in the transitional zone and in the upper radial zone, as indicated also by the anisotropy coefficient (Fig. 5). Furthermore, the chondrocytes are generally more rounded in shape than at 1 month. The overall height of the articular cartilage layer has also decreased (Figs. 3 and 7).

THREE MONTHS AFTER BIRTH

At 3 months, a more dramatic change in the structural organization of the articular cartilage layer is apparent [Fig. 3(C)]. Individual chondrocytes are highly oriented in space. Indeed, in the transitional zone and in the upper and lower radial zones, the anisotropy coefficient of the cells is comparable to that of chondrocytes in mature tissue (Fig. 5). In the calcified cartilage region, the horizontal as well as the longitudinal septa are mineralized, and the zone is now continuous [Fig. 4(B,C)]. The activity of the vascular invasion front is lower, and the overall height of the articular cartilage layer has undergone a further decrease (Figs. 3 and 7).

FOUR TO 7 MONTHS AFTER BIRTH

During the fourth to the seventh months, no noteworthy changes in the structural organization of the articular cartilage layer occur. By the third month, New Zealand white rabbits have entered puberty. Hence, maturation of the articular cartilage layer corresponds with sexual maturity. The adjacent growth plates, which are responsible for the longitudinal growth of the diaphysis and the metaphysis, remain fully operative until complete skeletal maturity is attained at 8 months. No significant changes in articular cartilage height occur during this fourth to seventh month period (Fig. 7).

EIGHT MONTHS AFTER BIRTH

At 8 months, New Zealand white rabbits are skeletally mature. The structure of the articular cartilage layer at this juncture [Fig. 3(D)] does not differ fundamentally from that manifested at 3 months [Fig. 3(C)], by which time the growth-plate-like activity of this layer has virtually ceased.

ULTRASTRUCTURAL ARCHITECTURE

The macromolecular architecture of the intercellular matrix compartments changes in parallel with the process of cellular reorganization, from a highly isotropic structure after the first postnatal month [Fig. 6(A)] to a highly anisotropic one after 3 months [Fig. 6(B)] up to 8 months [Fig. 6(C)]. The intercellular matrix compartments are defined according to the arrangement of the collagen fibrils. One month after birth, these fibrils are arranged randomly (isotropically) throughout the entire extracellular space [Fig. 6(A)] in all zones. By the end of the third month, the collagen fibrils are organized as in adult articular cartilage [Fig. 6(C)]. In the pericellular and territorial matrix compartments, the fibrils are arranged in a basket-like fashion around the cells...
and cell groups (chondrons). The pericellular matrix is rich in precipitated proteoglycans and contains numerous small cell processes (microvilli), which extend into the territorial matrix. In the interterritorial matrix compartment, which constitutes the bulk of the extracellular space, the collagen fibrils run parallel to each other in a predominantly longitudinal direction [Fig. 6(B)].

OVERALL HEIGHT OF THE ARTICULAR CARTILAGE LAYER

The overall height of the articular cartilage layer decreases precipitously between the first and the third postnatal months, at which latter juncture the rabbits enter puberty (Fig. 7). Between the third and the eighth months, there is no further significant change in this parameter (Fig. 7).

HEIGHT OF THE SUPERFICIAL (STEM-CELL) ZONE

The height of the superficial zone, which was defined on the basis of cell morphology, was found to be 1/13th of the overall height of the articular cartilage layer. It was also measured directly on autoradiographs of 3H-thymidine-labelled tissue sections (as the distance between the articular cartilage surface and the upper border of the labelled transitional zone). At each monthly juncture, these measurements corresponded well with the heights determined according to morphological criteria.13

DAILY GROWTH RATE OF THE EPIPHYSEAL BONE

The performance of the articular cartilage layer as a growth plate is reflected in the daily growth rate of the epiphyseal bone (Fig. 8). This parameter declines precipitously between the first and the third postnatal months; and by the fourth month, growth activity has ceased altogether (Fig. 8).

NET MONTHLY LENGTH-GAIN IN THE EPIPHYSEAL BONE

The total bone-length-gain achieved during each of the first 5 postnatal months decreases with time (Fig. 9). However, during the growth phase (i.e., the first 3 postnatal months), the monthly gain in bone length exceeds the height of the articular cartilage layer from which that of the superficial zone has been subtracted. This finding indicates that immature articular cartilage does not become reorganized by a process of internal tissue remodelling. If this were the case, the total length-gain in the epiphyseal bone per month during the growth phase would be smaller than the
corresponding height of the articular cartilage layer (after subtracting that of the superficial zone). Hence, immature articular cartilage must be completely resorbed and replaced by new tissue.

**IDENTIFICATION OF THE STEM-CELL POPULATION AND OF THE SITE OF RAPID CLONAL EXPANSION FOR TISSUE NEOFORMATION**

The location of the slowly proliferating stem-cell pool [estimated cycling time $\approx 12$ days (unpublished data)] was identified immunohistochemically after administering bromodeoxyuridine to the rabbits on a daily basis (via the drinking water) during the last 12 days preceding sacrifice. This analysis revealed a positive reaction within all cell nuclei of all zones, including the superficial zone [Fig. 10(A)]. Hence, the slowly proliferating stem-cell pool is located within the superficial zone, in analogy to the resting zone of a true growth plate.

Short-term labelling of tissue with $^3$H-thymidine tags only the rapidly proliferating cell populations. Autoradiography revealed a positive reaction only in the transitional zone and in the upper radial zone [Fig. 10(B)]. Hence, it is in these zones that the cells undergo the rapid proliferation and clonal expansion required for the neoformation of articular cartilage tissue and bone growth.

In summary, our data reveal that the postnatal (surface) growth-plate-like activity of the articular cartilage layer decreases precipitously after birth; by the fourth month (i.e., after puberty), it has ceased altogether. In contrast, the activity of the true (metaphyseal) growth plate is sustained until the eighth month, when the animals attain skeletal maturity. Hence, the bone-growth activities of these two structures must be regulated by different mechanisms. Our data further reveal that the reorganization of immature into mature articular cartilage involves the resorption and replacement of all articular cartilage zones except the most superficial (stem-cell) one. It is not based on a process of internal tissue remodelling.

**Discussion**

That the articular cartilage layer functions as a surface growth plate during postnatal development is well known. However, the growth mechanism and the bone-growth performance have not hitherto been analysed and quantified. Using the New Zealand white rabbit as our animal model, we have shown that the epiphyseal bone of the femoral condyles undergoes tremendous longitudinal growth in a radial direction. This growth activity peaks just 1 month after birth. Thereafter, it decreases continuously, and ceases altogether between the third and fourth postnatal months (by which time these animals have attained sexual maturity). The height of the articular cartilage layer decreases in parallel with the decrease in the rate of longitudinal bone growth. This finding is surprising for a structure that is functioning as a growth plate. In a previous study of ours, in which the height of the rat tibial epiphyses was compared with bone elongation, no relationship existed between these parameters. The growth activity of the metaphysis and of the diaphysis was regulated by modulations in the cell-cycle times and in the degree of cell hypertrophy; not by any change in the height of the growth plate as to render any direct regulatory interactions between these structures unlikely, in contrast to the situation existing in a foetal bone anlage. It thus appears that the endocrine regulation of the articular cartilage layer and of the adjacent growth-plate cartilage differs. Indeed, during the course of postnatal development, the articular cartilage layer becomes topographically so far removed from the true growth plate as to render any direct regulatory interactions between these structures unlikely, in contrast to the situation existing in a foetal bone anlage. Hence, it is conceivable that, during postnatal development, the growth activities of the articular cartilage layer and of the growth plate proper are hormonally regulated by different mechanisms and at different levels.
Our morphological data demonstrate that the articular cartilage layer undergoes a process of fundamental reorganization during the postnatal period. Hitherto, the mechanism underlying this structural remodeling has not been elucidated. It could involve either an internal reorganization of the tissue, or the resorption of all zones except the superficial (stem-cell) one and their replacement by appositional growth from the latter. We wished to elucidate which of

![Comparison of the Net Monthly Length-Gain in the Epiphyseal Bone with the Height of the Articular Cartilage Layer](image)

Our morphological data demonstrate that the articular cartilage layer undergoes a process of fundamental reorganization during the postnatal period. Hitherto, the mechanism underlying this structural remodeling has not been elucidated. It could involve either an internal reorganization of the tissue, or the resorption of all zones except the superficial (stem-cell) one and their replacement by appositional growth from the latter. We wished to elucidate which of

![Fig. 9. Bar graph comparing the net monthly length-gain in the epiphyseal bone (black columns) with the height of the articular cartilage layer from which that of the superficial zone has been subtracted (grey columns) at each postnatal month. During the growth phase (i.e., the first 3 postnatal months), when the articular cartilage layer is undergoing structural reorganization, the net length-gain in the epiphyseal bone exceeds the height of the articular cartilage layer. This finding indicates that the articular cartilage layer is structurally reorganized not by a process of internal remodeling, but by the resorption of all zones except the superficial (stem-cell) one and their neoformation by appositional growth from the latter. If a process of internal remodeling were involved, the height of the articular cartilage layer (excluding the superficial zone) would exceed the net monthly length-gain in the epiphyseal bone during the growth phase. Mean values (±S.E.M.) are represented.](image)

![Fig. 10. Light micrographs of cryosectioned articular cartilage tissue derived from the medial femoral condyle of 2-month-old New Zealand white rabbits, which had been administered either bromodeoxyuridine (via the drinking water) (A) or ³H-thymidine (by intra-articular injection) (B) 12 days or 1 and 2 days prior to sacrifice, respectively. In (A), the cryosection has been immunostained (brown coloration) to locate the origin of the slowly proliferating stem-cell pool. Since all cell nuclei within each of the zones are positively stained, the slowly proliferating stem-cell pool must be located in the uppermost, namely, the superficial zone. Daughter cells arising from the division of these stem cells feed a pool of rapidly proliferating chondrocytes which is responsible for rapid clonal expansion. This rapidly proliferating pool of cells was revealed by autoradiography for ³H-thymidine (B). Positive cell nuclei (dark-blue spots) were detected only in the transitional and upper radial zones. Hence, it is in these regions that the rapid clonal expansion necessary for bone elongation occurs. The cryosection in (B) has been stained with Toluídine Blue O to reveal all cell nuclei. SZ = superficial zone; TZ = transitional zone; URZ = upper radial zone. Scale bars: A = 30 μm; B = 15 μm.](image)
these alternative mechanisms is operative. Given that the daily rate of bone-growth activity peaks at a very early stage (1 month after birth), it is somewhat surprising that the articular cartilage layer does not become reorganized sooner, since we know from the physiologist that a highly anisotropic columnar organization of the cells is required to achieve the highest axial growth rates. Indeed, clinical conditions that are characterized by a reduction in the anisotropic organization of the cells of the growth plate, such as achondroplasia, are associated with a significant depression in the growth rate. Our finding may reflect the fact that the epiphysis initially enlarges not only in a longitudinal direction (which is exclusively the case for the metaphysis and the diaphysis), but also radially and laterally (Fig. 1(A)), and in an irregular manner to produce the different radii of the hemispherical structures. As the shaping (modelling) process approaches completion and the growth activity of the epiphyseal bone declines, the articular cartilage tissue may only then begin to assume a more mature anisotropic structure because the lower growth activity is directed in a more perpendicular direction. The existence of well-organized cell columns at this stage is, in fact, not in doubt. Their presence depends on the degree of tissue maturity attained and on the local topographical needs to be satisfied. A mature, structurally well-organized (anisotropic) articular cartilage layer is characterized by a higher mechanical stiffness (Young’s modulus) than is its immature foetal counterpart, in which the cells and extracellular macromolecules are more isotropically organized.

Labelling of the slowly proliferating stem-cell pools with bromodeoxyuridine revealed these to be located in the superficial zone. Labeling of the rapidly proliferating (i.e., transit-amplifying) cells with 3H-thymidine disclosed these to occur in the transitional zone and in the upper radial zone. The superficial zone also supplies the stem cells that effect the lateral expansion of the articular cartilage layer. Hence, the daughter cells that move vertically downwards and feed the rapidly proliferating pool of transit-amplifying cells in the transitional and upper radial zones effect longitudinal growth, whereas those that are displaced horizontally and remain confined to the superficial zone replenish the stem-cell pool and effect lateral growth (Fig. 1(B)). This bidirectional process of stem-cell replication is reminiscent of the horizontal and vertical delivery of daughter cells for the growth of the skin and the ocular lens. Thus, in analogy to the true growth plate (i.e., at the articular cartilage/epiphyseal growth plate) and to the articular cartilage layer in marsupial models, the superficial zone consists of a slowly proliferating, self-renewing population of stem cells, which not only effect a lateral expansion of the articular cartilage layer, but also supply the rapidly dividing, transit-amplifying pool for rapid clonal expansion and hence for elongation of the epiphyseal bone. Lengthening of the epiphyseal bone is also achieved by the hypertrophy of cells in the lower radial zone, the mechanism being similar to that whereby the metaphyseal growth plate affects axial bone growth.

Despite its dual functionality as a surface growth plate for radial growth of the epiphyseal bone and as an articulating sheet, the articular cartilage layer operates as an integral unit, with a continuum of cell activities from a “resting” phase in the superficial zone (slowly cycling stem cells), through one of high proliferative activity in the transitional and upper radial zones (transit-amplifying cells), to hypertrophy and mineralization in the calcified cartilage zone (terminal differentiation). Finally, the cartilage tissue is resorbed and replaced by primary bone trabeculae, in analogy to a true growth plate.

Our data relating to the height of the articular cartilage layer, to its bone-growth performance, and to the absolute elongation of the epiphysis, indicate that immature cartilage is subject to the complete resorption of all zones except the superficial (stem-cell) one and their replacement by oppositonal growth from the latter. Such resorptive activity does of course occur also in a “true” growth plate (i.e., in the physis). However, in the latter case, the epiphyseal cartilage is replaced by bone without being itself replenished, whereas immature isotropic articular cartilage not only effects bone growth, but is itself replaced by mature anisotropic articular cartilage. Hence, immature articular cartilage does not become reorganized by a process of internal tissue remodelling. It is first destroyed by the resorption front of ingrowing epiphyseal blood vessels, which spares only the superficial zone, and is then completely replaced after the activation of stem cells in the latter. Consequently, the remodelling of articular cartilage tissue from an isotropic to an anisotropic structure does not occur at a fixed topographical position in space, but by a growth process that involves an elongation of the underlying bone.

This finding has potentially important implications for the engineering of articular cartilage tissue in adult organisms. Adult articular cartilage lesions would be ideally repaired by tissue that manifested a high degree of structural anisotropy from the very onset of the healing process, in order to ensure its longevity and mechanical competence. But currently, cell-based and other therapeutic approaches involve the implantation of immature cartilage manifesting a random cell distribution. Since our findings demonstrate that mammalian articular cartilage is not physiologically reorganized if a fixed topographical position in space, it is very unlikely that these current tissue-engineering approaches will lead to optimal repair results. In the light of our findings, these architectural aspects should now be given due consideration during the planning of functional tissue-engineering approaches to cartilage repair in adult mammalian organisms. Our study has revealed the postnatal development of articular cartilage to be a complex process. Although the layer functions as a surface growth plate for the epiphysis during this phase, the growth activity is probably not regulated in an analogous manner to that in the metaphyseal/diaphyseal growth plate.

References


