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MINERALIZATION OF NORMAL AND RACHITIC CHICK GROWTH CARTILAGE: VASCULAR CANALS, CARTILAGE CALCIFICATION AND OSTEOGENESIS

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Abstract

This paper reviews recent work in the authors' laboratories that has led to new observations and thoughts concerning the mineralization of normal and rachitic chick growth cartilage. The proximal tibial growth cartilages of normal and rachitic chicks were rapidly frozen and prepared for SEM and biochemical studies. Using a scanning microfluorimetric technique we showed that at the mineralization front of normal and rachitic cartilage there is an abrupt change in chondrocyte metabolism. Thus cells in this region exhibited an increase in NADH and oxidative metabolism. In rickets, there was a decrease in the reduced pyridine nucleotide content of each of the zones. The reversal in chondrocyte metabolism was not due to low oxygen tension. SEM observations indicated that this region of cartilage was well supplied with vascular channels; moreover, mineral was first seen deposited in matrix in close proximity to the blood supply. Indeed these vascular channels appeared to be a basic architectural feature of normal cartilage, although disorganized in the rachitic state. The morphological studies also showed that gaps existed in the continuity of the mineral phase in normal cartilage. Although the rachitic cartilage does mineralize, discontinuities in the mineral distribution are much more severe, with the general failure of fusion of adjacent mineral clusters. These structures would serve as pathways for transport of nutritional factors and gases to chondrocytes that are distant from the vascular channels. Observation of hypertrophic cells reinforced the view that some osteoblasts represented a terminal stage in the maturation of chondrocytes.

KEYWORDS: Chick growth cartilage, mineralization, hypertrophic chondrocytes, rickets, bone disease, vitamin D, energy metabolism, redox, oxygen, NADH, microfluorimetry.

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Introduction

For progress to be made in understanding the mechanisms of biological mineralization, observational studies need to be closely coupled with underlying biochemical events. Cognisant of the importance of this approach, work in our laboratories has been aimed at mapping ionic, elemental and energy dependent changes that take place during the mineralization of the growth cartilage (Boyde and Shapiro 1980, Shapiro and Boyde 1984, Shapiro et al 1982, Kakuta et al 1985).

The techniques that we have utilised preserve the metabolic state, limit ion redistribution and maintain the morphological integrity of the tissue. Using these techniques we have shown that at the site of mineral formation there is a profound change in cell redox state and a dramatic decrease in the energy charge ratio (Shapiro et al. 1983). This decrease in oxidative activity correlates with the observation that there is a profound drop in the oxygen tension in the late hypertrophic zone (Brighton and Heppenstall 1971). Indeed, Brighton and Hunt (1974) considered that the low oxygen tension inhibits oxidative phosphorylation and thereby stimulates mitochondrial ion efflux. These workers believed that uptake of these ions by the extracellular matrix provided the driving force for the initiation of mineralization. We have also used these mapping techniques to show that following formation of a calcified matrix, the hypertrophic chondrocyte remains a viable constituent of both cartilage and bone (Boyde and Shapiro 1980, Shapiro and Boyde 1984).

The level of oxidative metabolism by cells of the growth cartilage is dependent on the available oxygen supply, which in turn must depend on the vasculature. It is now clear that significant differences exist in the blood supply to the mammalian and avian cartilages. In excellent studies of the vascular canals in chick growth plate cartilage, Howlett (1979) and Howlett et al. (1984) indicated that these canals pass through calcifying cartilage into the hypertrophic zone. Some of these channels can also be seen in the proliferative zone. Our own recent studies of the normal and rachitic growth

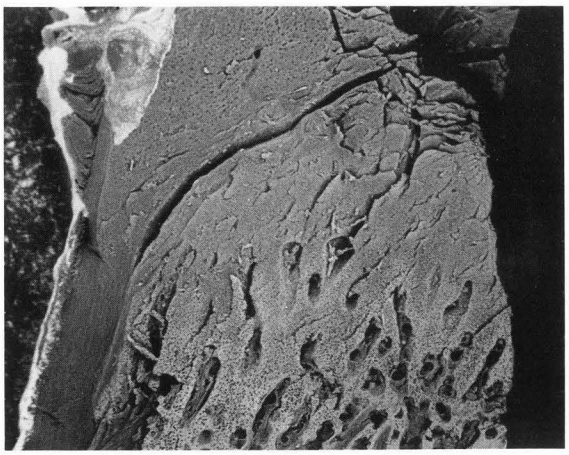

Figure 1. Longitudinal view of normal growth cartilage of the chick after microfluorimetric scanning. The sample was prepared by rapid freezing in freon cooled with liquid nitrogen. Prior to SEM, the tissue was dehydrated with acetone, critical point dried and coated with gold. 25 kV SE image. Fieldwidth = 4360 μm.

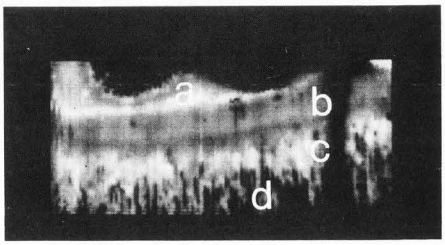

Figure 2. Microfluorimetric scan of NADH in growth cartilage. 4 zones can be seen. Zone **a** is non-specific fluorescence associated with hyaline cartilage. The dark band **b** indicates a zone of high oxidative activity corresponding to the region of cell proliferation. The white zone **c** is in hypertrophic cartilage and the fluorescence indicates high levels of NADH. Note the sharp border between these two zones. The black zone **d** below hypertrophic cartilage indicates a high degree of mitochondrial activity, this zone corresponds to calcified cartilage - bone. Fieldwidth = 16mm.

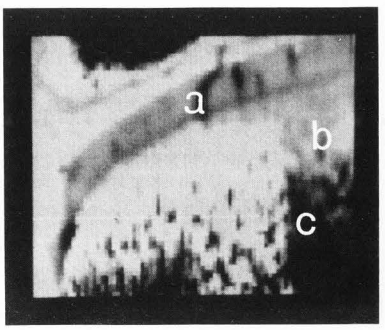

Figure 3. Microfluorimetric NADH scan of rachitic cartilage. Note the very low contrast levels in each of the zones. The darkest zone is in proliferative cartilage **a** while the extensive white zone corresponds to hypertrophic cartilage, **b.** Despite the low level of pyridine nucleotides sharp borders still exist between proliferative and hypertrophic cartilage. Zone **c** contains bone and calcified cartilage. Fieldwidth = 18 mm.

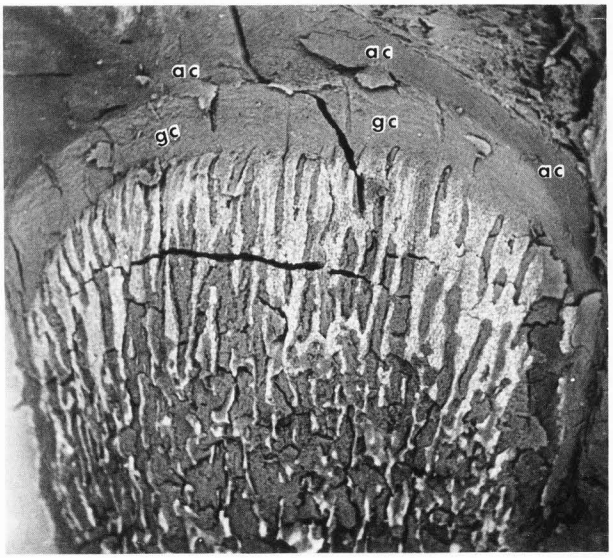

Figure 4. Longitudinal freeze fracture through proximal head of chick tibia. Mineralizing front in cartilage crosses above centre of field. gc = non-mineralized growth cartilage. ac = articular cartilage. Black features are artefact cracks. 20 kV BSE image. Fieldwidth = 3500 μm.

cartilage of the chick provide important new information relevant to the oservations described above (Boyde and Shapiro, in press). Results of these studies address the following issues: (i) whether changes in redox and energy metabolism at the mineralization front are controlled by the local oxygen tension: (ii) whether chondrocytes can alter their phenotype and form bone.

Measurement of the Energy Status of Cartilage

The energy state of a cell can be assessed in a number of different ways. For example, we have measured the actual concentration of the adenine nucleotides using high pressure liquid chromatography (Shapiro et al 1983). We have also determined the levels of redox-sensitive components such as NAD and NADH using an enzyme cycling technique (Shapiro et al 1982). Probably the best approach to evaluating energy metabolism is by scanning microfluorimetry, using the system described by Quistorff et al (1985) and applied to hard tissues by Chance and our group (Shapiro et al 1983). The basis of the system is that

mitochondrial activity is reflected in the redox state of the respiratory chain. Thus, cells that exhibit a high level of oxidative activity contain a low level of reduced NAD and high levels of oxidized flavoprotein. In contrast, cells that are in a reduced state have high NADH and low oxidised flavoprotein levels. The concentration of both of these components can be measured by their unique fluorescence at a low temperature.

To measure these components, tissue must be collected using conditions that trap the energy state of the respiratory chain. In practical terms, immediately following the death of the chick, the proximal tibial head is exposed, incised in a longitudinal direction and immersed in CCl_2F_2 cooled in liquid nitrogen (-155^{o}C). The sample is stored frozen in liquid nitrogen.

Freeze fractured fragments of cartilage are milled to produce a plane surface and three small holes are drilled into the tissue to serve as fiducial points. These holes facilitate subsequent correlation of the fluorescent output with the tissue SEM morphology. To generate a scan, a dedicated microprocessor directs a light guide containing a glass fibre optic system (20-100 μm diameter) connected to a time sharing fluorimeter across the specimen in a raster of points. At each point, fluorescent light (NADH 455 nm) is collected, and the signal both displayed on a TV monitor and stored in a computer. Once scanned, the sample is dehydrated, critical point dried, coated with carbon or gold and viewed in an SEM. Evidence for the formation of mineral is obtained by inspection of the BSE-SEM image or EDX microanalysis. The drill holes are then used to relate the morphological structure to the redox map (Fig.1).

Normal growth cartilage contains areas of high and regions of low fluorescence (Fig. 2). High fluorescence (white) is due to the presence of NADH and it indicates sites of low oxidative activity. In contrast, low fluorescence (black), due to low levels of NADH, is indicative of high mitochondrial activity. When correlated with the morphology of the zone, it is clear that the proliferative zone of the chick cartilage exhibits a high level of oxidative activity, whereas in the hypertrophic zone there is a sharp redox change and energy metabolism is depressed. We have commented before that similar types of sharp borders are seen in tissues that have been rendered hypoxic or ischemic (Shapiro et al 1982). This observation is pertinent to an earlier study of growth cartilage metabolism where it was pointed out that there is a dramatic decrease in the oxygen tension at sites of mineralization (Brighton and Heppenstall, 1971).

The scanning technique has also been used to map redox changes in the rachitic growth cartilage. A scan of the tissue (Fig.3) shows a low level of reduced pyridine nucleotides and hence the definition of each zone is weak (the details of this experiment are described in detail elsewhere, (see Kakuta et al. 1985). Nevertheless, in the longitudinally very extensive calcified cartilage regions with bone forming on cartilage, sharp borders are seen and hence it must be assumed that changes in oxygen tension regulate mitochondrial oxidative activity and, eventually, mineral formation. Clearly, the oxygen tension and the vascularity of the tissue play a key role in regulating the calcification of the growth cartilage. The importance of these factors will be further considered in the next section.

Vascularity of the Growth Cartilage

We have recently made detailed SEM observations of the morphology of the chick growth cartilage, having paid particular attention to the vascular canals penetrating this region and the consequential interesting features of the mineralization process. These studies are described in detail elsewhere (Boyde and Shapiro, in press) and will only be reviewed briefly here.

The architecture of the chick growth cartilage is shown in Figs. 4, 5 and 6, which are low power views of the proximal end of the normal chick tibia, freeze fractured in the long axis of the bone. In Fig. 4, which was freeze dried, the longitudinal vascular channels show as grey in the generally white mineralized cartilage zone. They arise from metaphyseal vessels, and many of them penetrate the growth cartilage (gc): some penetrate as far as the articular cartilage (ac). In Fig. 5, the sample has been made anorganic, so that only fully mineralized portions of the cartilage and bone are retained. The full extent of these canals is revealed in a transverse fracture plane through the mineralized cartilage, as shown in Fig. 6. It is evident from specimens like these that the tissue is not just well supplied with vascular canals, but that the cartilage is organised around these structures.

Details of Mineralization

Other interesting aspects of these canals concern what happens at the mineralizing front region. If we look at deproteinised specimens end on (Figs.7 & 8), we see the shape of this front. Although the cartilage matrix layer next to the canal complexes does not mineralize, the level of mineralization is most advanced nearest to the canals (Fig. 7). Thus, these regions stand above the general front of mineralization.

The concept of a "front" needs to be defined, however. At higher magnifications, we can see how aggregates or agglomerates of mineral particle clusters finally fuse when they get close enough (Fig. 8). Unfused clusters exist above the front: these are lost in the preparative process.

The relative advance in degree of mineralization in the cartilage nearest to the canals is true in another sense and can be appreciated in another way, by examining the tissue density using backscattered electron (BSE) imaging. Using polymethyl-methacrylate (PMMA) embedded samples, we can reduce topography to minimal levels by careful polishing technique, when the (non-directional) BSE signal intensity is proportional to the amount of mineral deposited within the tissue. Section surfaces from within the

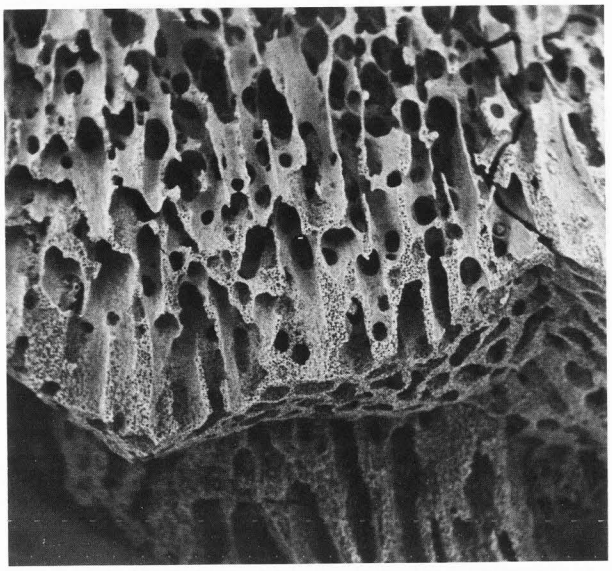

Figure 5. Similar specimen to Fig. 4 made anorganic by treatment in an Na_2O_2 solution, showing longitudinal and transverse break planes. Vascular canals penetrate the cartilage in a longitudinal direction. 20 kV BSE image. Fieldwidth = 5000 μm.

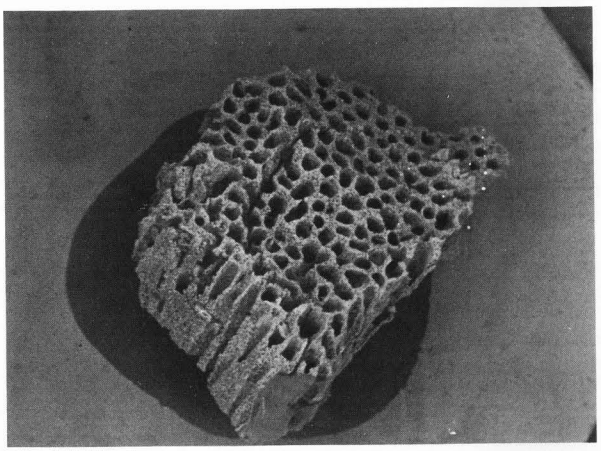

Figure 6. Transverse fracture through the centre of the mineralized cartilage zone (no bone at this level) in a similar anorganic specimen. 20 kV BSE image. Fieldwidth = 4000 μm.

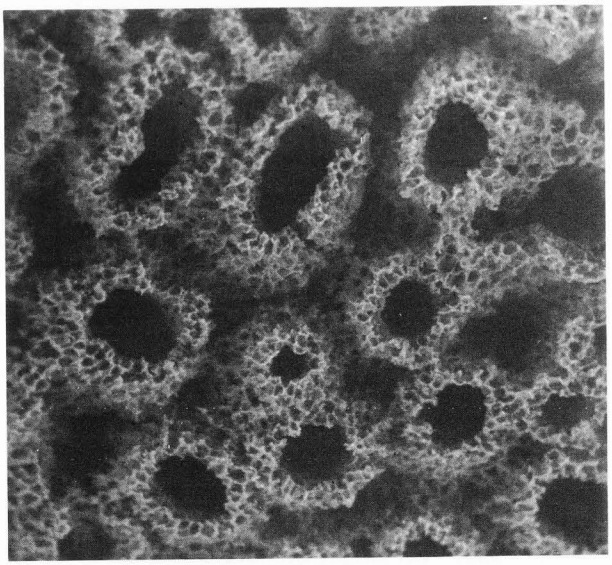

Figure 7. Anorganic specimen prepared by dissolving organic matrix in a sodium hypochlorite solution, viewing the mineralizing front in the proximal head of a chick tibia. The level of the front is highest next to the vascular canals, which appear as hollow tubes in this preparation. 20 kV BSE image. Fieldwidth = 725 μm.

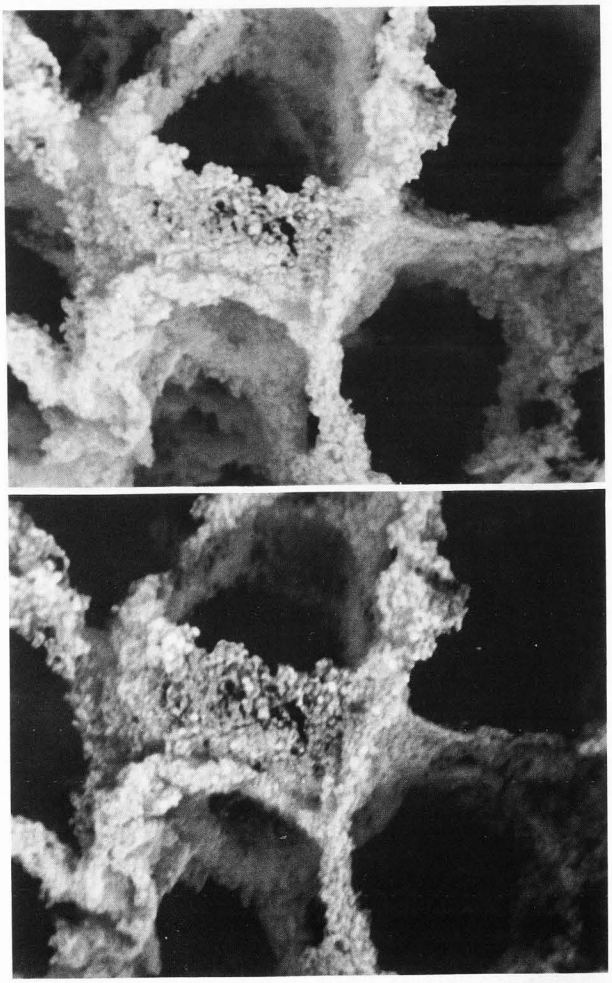

Figure 8. Stereo-pair, 10 kV BSE images, 10^{o} tilt. Mineralizing front prepared by plasma ashing and washing with ethanol to disperse fine ash. Fieldwidth = 40 μm.

mineralized tissue show that the degree of mineralization is highest in the matrix nearest to the canals, and persists at a lower level in the matrix most remote from the canals (Fig. 9).

Initiation of Mineral Formation

It is important to relate information on the vascularity of both the rachitic and the normal growth cartilage to established views concerning the initiation of mineral formation. We question the hypothesis that a decrease in oxygen tension triggers the mineralization process. Our reasons are as follows: First, it is highly unlikely that cells are hypoxic in the calcifying region. As both hypertrophic and calcifying cartilage are well supplied with vascular channels, it is more likely that cells in this region are in a normoxic state. Second, if mineralization is associated with a change in energy metabolism, then the signal for this change may well be related to the presence of the vascular canals and not to their absence. How these two events are linked needs to be explored. Third, studies of normal and rachitic cartilage suggest that mineralization is related closely to chondrocyte maturation rather than to local changes in oxygen tension or even tissue vascularity. Thus, the redox change at the calcification front may be due to a change in phenotypic expression, related to the maturation of chondrocytes and not to environmental factors.

Diffusion through Mineralized Cartilage

The presence of the vascular canals would ensure an adequate supply of oxygen to the surrounding chondrocytes. How cells at a distance from the vascular canals receive nutrients and gases requires some consideration. It has been assumed that passage of these components is governed by simple diffusion characteristics. Hence, when calcification takes place, with sources of nutrients blocked, these cells would rapidly die.

Figures 10 and 11 show that this assumption may not be correct. Gaps can be seen in the continuity of the mineral phase in the walls of the calcified lacunae. These regions would serve as channels for the transport of tissue fluid through non-mineralized matrix windows to the cells most distant from the blood vessel complexes in the growth cartilage. Thus, even these cells would be provided with factors essential for chondrocyte survival in a calcified matrix. We have also noted that no cell in this region is more than 4 cell distances away from a nutritional canal (Shapiro and Boyde 1984).

Chondrocyte Vitality

Histological studies of the growth cartilage strongly suggest that following the formation of calcified cartilage, chondrocytes die and they are replaced by bone. This conclusion has been questioned in a number of reports (Holtrop 1972, Hanaoka 1976). Hunziger et al (1984) drew attention to difficulties in fixing the cells by conventional techniques. These workers showed that if the tissue was processed using a high pressure freezing method, then there was minimum evidence of cell necrosis. Our earlier studies support this conclusion (Boyde and Shapiro 1980). Measurement of elemental levels in epiphyseal chondrocytes isolated from the most calcified regions of the cartilage show that these cells maintain normal levels of potassium and other elements. If the hypertrophic chondrocyte remains viable, is there a new role or function for this cell in the osteogenic phase of endochondral bone formation? One possibility is that the chondrocyte changes its morphology and functions as an osteoblast.

Some evidence to support this possibility is observational and shown in Fig. 12 in a section through the calcified cartilage-bone region of the growth plate. The tissue has been embedded in plastic and imaged by BSE. Bone (grey) is less calcified than the surrounding more densely mineralized (white) cartilage. The figure shows that bone is deposited within chondrocyte lacunae, and in some lacunae spaces for two osteoblasts can be seen. While caution must be exercised in interpreting these observations, they increase the power of other pertinent findings. These findings include a report that late hypertrophic chondrocytes are capable of proliferating (Weiss and Silberman 1986) and some elegant studies by Von Der Mark and Mollenhauser (1986), who demonstrated that hypertrophic chondrocytes could switch synthesis from type II (cartilage) to type I (bone) collagen.

These morphological and biochemical observations cannot be ignored. Together they lend support to the view that hypertrophic chondrocytes are not a terminal stage of development. We suggest that even at this apparently late stage, chondrocytes are capable of cell division and can express a phenotype more closely associated with bone than cartilage.

Rickets

We have also carried out an evaluation of the vascularity of rachitic growth cartilage. Our findings clearly indicate that while the longitudinal vascular channels are more disorganised those present in the normal cartilage, the tissue is very well vascularised. In the vitamin D deficient state, mineralization does occur and is also seen to commence first around the vascular canals, then spreading to contiguous matrix. However, the continuity of the crystals of the mineral phase is deficient. Thus the clusters fail to fuse and the entire calcified matrix remains soft.

Acknowledgements

The authors wish to thank Elaine Maconnachie for SEM assistance. These studies were supported by funds from the MRC, the SERC and the NIH (DE 02623 and AM 34411). This paper was presented at the Edward J Reith Memorial Symposium on the Scientific Investigation of Mineralized Tissues using SEM at New Orleans, May 5 - 7, 1986.

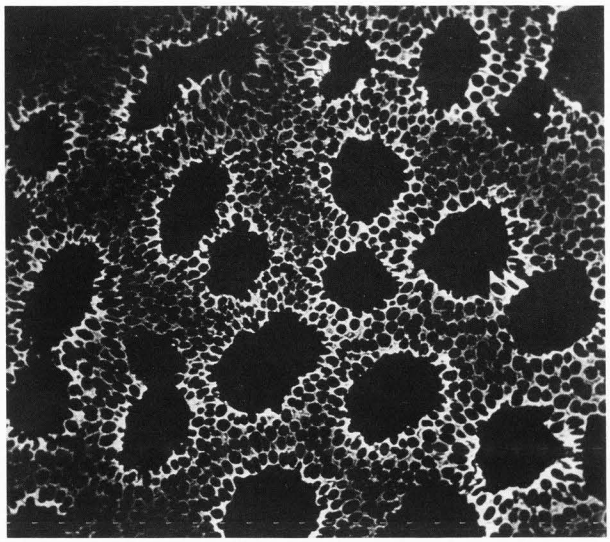

Figure 9. 20 kV BSE image of polished surfaces of PMMA embedded block of normal chick cartilage. A transverse section through mineralized cartilage (white), Degree of mineralization (whiteness) is greater next to the vascular canals (the large black areas). Chondrocyte lacunae show as smaller black areas. No bone present at this level. Fieldwidth 900 μm.

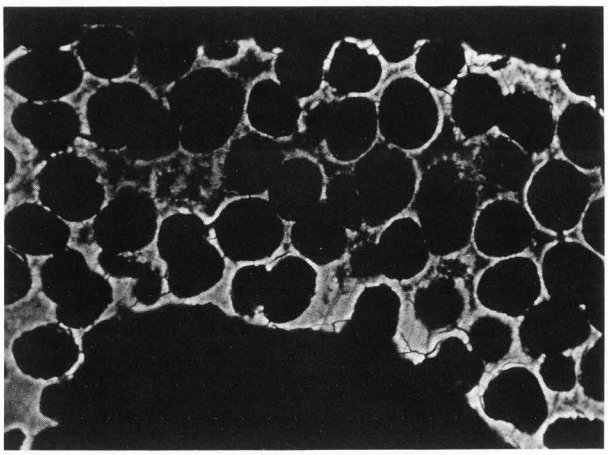

Figure 10. Similar preparation at slightly more distal level. Note that the chondrocyte lacunae, as seen in this image of the distribution of the mineral phase, are partially confluent. Further, the mineralization in some thicker areas of matrix is patchy owing to failure of fusion of adjacent agglomerates (calcospherites). Fieldwidth = 180 μm.

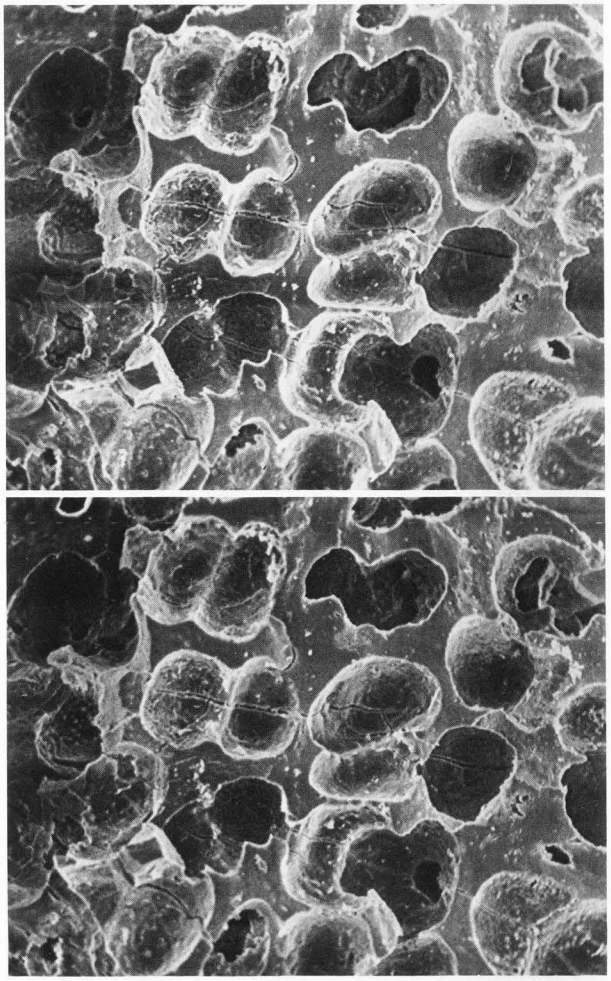

Figure 11. Stereo-pair, 10 kV SE images, 10° tilt. Freeze fracture through mineralized cartilage, then made anorganic: showing confluence of adjacent chondrocyte lacunae via windows in the otherwise continuous mineral phase distribution. Fieldwidth = 83 μm.

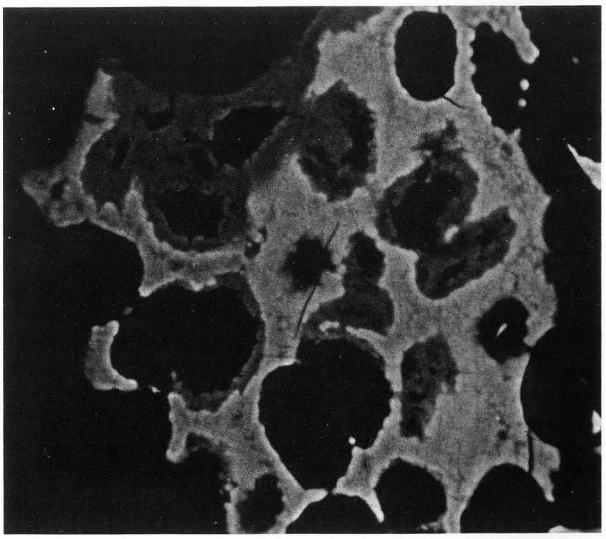

Figure 12. 20 kV BSE image of longitudinal section surface through PMMA embedded chick tibia proximal head growth plate at level where bone (grey phase) is deposited on cartilage (whiter phase) and in the chondrocyte lacunae. Fieldwidth = 90 μm.

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Dedication

We dedicate this contribution to the memory of our dear friend and colleague, Prof Edward J. Reith, in whose honour the symposium was organised at the New Orleans SEM meeting in May 1986.

Discussion with Reviewers

B.D. Boyan: Since in vitro chondrocytes are usually cultured in the presence of serum, serum factors may be involved in the loss of type II collagen and the synthesis of type I collagen observed by Weiss and Silberman. Thus the cells may not actually be osteoblasts but chondrocytes with a twist. Your observations that vascular channels extend into the hypertrophic region indicates that these cells might also be exposed to serum factors in vivo. Would you comment on this?
Authors: Our speculation that the chondrocytes may become osteoblasts is based entirely on morphology. It is very easy to recognize bone from cartilage. In the SEM case, this is done on the basis of the size of the lacunae in the mineralized matrix (i.e., the size of the cells) and the characteristic density of the mineralized matrix. We cannot believe that bone would have been made by chondrocytes which were still chondrocytes.

These studies are further supported by the in vitro studies by Weiss and Silberman and by Von der Mark and Mollenhauser (1986). Von der Mark has published extensively on this topic over the last 10 years indicating that the phenotype of the cartilage itself can be modified by external environment. Clearly these types of experiments would indicate that one cell type has the ability to express proteins which are characteristic of another cell type. We are therefore suggesting that there is a change in expression in the course of chondrocyte differentiation and associated with this change in expression there is the formation of bone.

B.D. Boyan: Wuthier and others have reported that vitamin D appears to alter the proliferative zone of cartilage of vitamin D-dependent rachitic chicks. Do your observations agree with these findings?
Authors: In our SEM study of the morphology of the epiphyseal growth cartilage rachitic chicks, we also found a dramatic change in the morphology of the proliferative cartilage zone. The regular arrangement of the chondrocytes was disturbed as well as the orientation of the blood vessel canals. Nevertheless, the tissue was well vascularized and mineralization - to a limited degree did occur. The findings are described in full in Boyde and Shapiro (1987).

J. Glowacki: These studies focus on some of the critical questions concerning the relationships amongst cartilage mineralization, vascularization, chondrolysis and the fate of the chondrocytes. Do the microfluorimetric scans reveal changes in chondrocyte energy status in relation to small invading capillaries?
Authors: In longitudinal scans it is very difficult to see localized changes in metabolic activity of cells in relationship to the blood vessels. Recent studies have shown that when the growth cartilage is sectioned transversely, and a higher resolution is achieved through the use of a narrower fiber optic diameter system (Haselgrove et al., in preparation), then the cells that surround the blood vessels are seen to place a greater reliance on oxidative energy metabolism than those remote from the vascular canals.

J. Glowacki: Much of the literature on the mechanisms and regulation of cartilage mineralization has relied upon vitamin D-deficient models wherein cartilage mineralization is delayed and reduced. It has been assumed that the abnormality of rachitic cartilage is due to the animal's hypocalcemia. Do your studies suggest any other contributing factors attributable to decreased levels of 1,25-dihydroxyvitamin D and direct effects upon monocytes, "prechondro-clasts", or cells of the immune system?
Authors: It is often assumed that the morphological changes and the biochemical changes are due to a simple change in the vitamin D status of the animal. However, this cannot be the case because vitamin D deficiency is also associated with profound changes in both the level of serum calcium and phosphate. Experiments are now in progress to study the energy status of these animals using the microfluorimetric and high pressure liquid chromatography techniques. We do not have, at this time, any information on the effects of 1,25-dihydroxyvitamin D on cells of the immune system or the monocyte macrophage series.

J. Glowacki: Is there any direct evidence for the diffusion of small molecules through calcified cartilage?
Authors: To answer this question requires that we define what we mean by calcified cartilage. The calcified cartilage tissue is actually very porous because there are large uncalcified fenestrae in the matrix (Figs. 10 and 11). It is therefore unnecessary for there to be a system whereby small molecules could diffuse through the mineralized matrix as such, but we cannot exclude this possibility. We also cannot imagine how we would set up an experiment in which we could test whether small molecules would diffuse through the calcified part because the calcified part is interspersed with non-calcified parts.