# Ultrastructural localisation of alkaline phosphatase activity in osteoarthritic human articular cartilage

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SUMMARY   The distribution of alkaline phosphatase activity in human articular cartilage from normal and osteoarthritic joints has been examined by an electron microscope technique, probably for the first time. In osteoarthritic cartilage chondrocytes and matrix vesicles close to the tidemark were positive for alkaline phosphatase activity. Large numbers of matrix vesicles were found within the extracellular matrix of osteoarthritic cartilage, and there is a specific relation between phosphatase activity, matrix vesicles, and initial mineral formation in the tidemark region of articular cartilage.

Osteoarthritis may be considered as a group of heterogeneous diseases with a final common pathway involving abnormal changes in some joint tissues and in particular articular cartilage. This 'joint failure' can be initiated by various endogenous processes, and the occurrence of hydroxyapatite and pyrophosphate crystals in joint fluids has led to the concept of crystal deposition arthropathy and apatite associated arthritis.[1–3] Previous biochemical studies indicated there may be abnormal calcification in human osteoarthritic cartilage as seen by increased amounts of alkaline phosphatase activity.[4 5] In addition, detailed electron microscope studies have shown several different types of calcium phosphate crystals in arthritic articular cartilage.[6–9] These apatite-like crystal types appear to be associated with matrix vesicles, which have been shown in other calcifying tissues to contain most of the alkaline phosphatase activity and to initiate the calcification process.[10 11]

Robison initially introduced the idea that alkaline phosphatase enhanced calcification of cartilage, probably by raising the phosphate concentration locally.[12] Gomori demonstrated histochemically that cartilage calcification occurred only in areas that were rich in alkaline phosphatase.[13] Since then alkaline phosphatase has been localised in the calcifying regions of bone[14 15] and teeth,[16] further substantiating Robison's view.

At the ultrastructural level alkaline phosphatase has been localised in a number of different calcifying cartilages. The distribution in growth plate has been described by Matsuzawa and Anderson,[17] Thyberg and Friberg,[18] Takagi and Toda,[19] and Ralphs and Ali[20]; in fracture callus by Gothlin and Ericsson,[21] Salomon,[22] and Volpin *et al*[23]; and in condylar cartilage by Meikle[24] and Lewinson *et al.*[25] In articular cartilage the enzyme distribution has been studied using a light microscope technique only.[26] It is intended to extend these observations to the ultrastructural level and look for associations between alkaline phosphatase activity, chondrocytes, matrix vesicles, and initial mineral formation in human osteoarthritic articular cartilage.

## Materials and methods

Fresh human osteoarthritic cartilage was obtained from femoral heads resected for total hip replacement. The cartilage was degenerate, fibrillated cartilage from the pressure bearing, progressive area. It was usually restricted to a fringe surrounding the eburnated bone around the superior surface of the femoral head. This cartilage is usually yellow and found as a sloping or shelving ring adjacent to the circle of eburnated bone (type IV cartilage, see Ali and Bayliss[5]). The specimens were obtained from five female patients aged 58, 61, 63, 69, and 76 years. In the last year it has been impossible to obtain fresh, age matched, normal human articular cartilage. Disease free cartilage was obtained from the femoral heads of two female patients aged 83 and 85 years with subcapital fractures of the neck of

femur. Small pieces of cartilage were excised down to and including the subchondral bone. Some of the specimens were put into 2·5% glutaraldehyde in 0·085 M sodium cacodylate buffer and then processed for transmission electron microscopy by standard techniques described elsewhere.[5]

For the visualisation of alkaline phosphatase activity a metal salt method similar to that described by Volpin et al[23] was used. Briefly, this consisted of taking very thin tissue slices (30–200 μm thick) through the whole depth of cartilage. These were cut with new razor blades under a dissecting microscope while immersed in 1·5% glutaraldehyde in 0·085 M sodium cacodylate buffer. After one hour the tissue slices were washed in buffer and immersed in the incubation medium (containing the enzyme substrate 9 mM sodium β-glycerophosphate, 10 mM magnesium chloride, 3·6 mM lead nitrate, and 40 mM trometamol (TRIS)/HCl buffer at pH 9) for 30 minutes at 37°C.

To test for false non-enzymatic deposition of reaction product control tissue slices were incubated in a medium to which 2 mM levamisole had been added as an inhibitor of alkaline phosphatase activity. Further control slices were incubated in a medium which did not contain the enzyme substrate (sodium β-glycerophosphate). After washing in 0·085 M cacodylate buffer for 10 minutes test and control cartilage slices were postfixed in 1% osmium tetroxide in cacodylate buffer for one hour. After dehydration through graded ethanols and finally 1:2-epoxypropane the specimens were infiltrated with Araldite resin overnight and polymerised at 60°C for 48 hours. For orientation purposes 1 μm thick sections were cut for light microscopy with glass knives and stained with Humphrey's stain. Thin sections (60–80 nm) were cut with diamond knives and collected on parlodion coated copper grids. Sections were examined, unstained or after staining with saturated alcoholic uranyl acetate for 30–45 minutes and lead citrate for five minutes, on a Philips EM300 at 60 kV or 80 kV.

## Results

### 'NORMAL' CARTILAGE FROM SUBCAPITAL FRACTURE SPECIMENS

Light and electron microscope sections showed the cartilage surface was smooth with collagen fibres in the superficial layer arranged parallel to the surface. Chondrocytes were discoidal with their long axes parallel to the cartilage surface. In the mid-zone cartilage chondrocytes were spheroidal and equally spaced or occasionally arranged in columns of four to eight cells. The collagen fibres in the mid-zone matrix were predominantly radial. In the deep zone

the calcifying front or tidemark region appears even or gently undulating by light microscopy or low power electron microscopy. At higher magnification matrix vesicles in the pericellular region of chondrocytes were present at all levels of the cartilage but were more frequent in the tidemark region. In this region microcrystals were present inside vesicles, and mineral nodules could be seen either forming from vesicles or lying next to them (Fig. 1). In many sections from several blocks of the normal cartilage, from subcapital fracture specimens, clusters of chondrocytes were frequently seen in this region, but only one cluster was positive for alkaline phosphatase activity (Figs 1 and 2). This reaction product appeared as a few electron dense beads along the chondrocyte cell membrane (Fig. 2, arrows). This cluster of chondrocytes was only about 40 μm from the calcifying front.

Chondrocytes and matrix vesicles further than 40 μm from the calcifying front showed no evidence of alkaline phosphatase reaction product in disease free articular cartilage. Lack of penetration of the substrate as a factor indicating lack of enzyme activity could be ruled out because some cartilage slices were only 40–50 μm thick and would be penetrated relatively easily by the substrate. Matrix
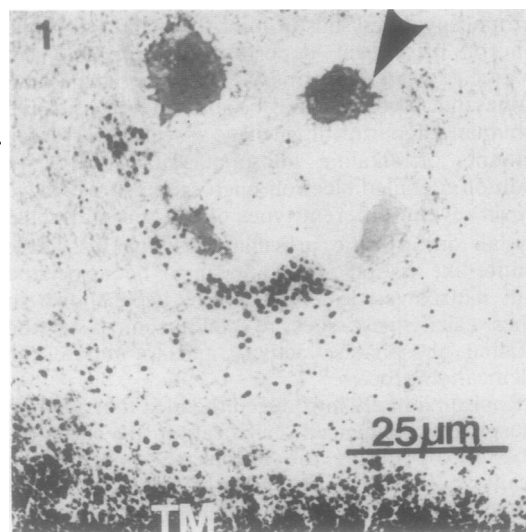


Fig. 1   Low power electron micrograph of a section of disease free cartilage (woman, 85 years) incubated for alkaline phosphatase activity. A cluster of partially reactive chondrocytes are seen approximately 40 μm from the tidemark (TM). This unstained section shows the distribution of mineral nodules around the cluster of chondrocytes. The chondrocyte indicated by an arrow head is shown enlarged in Fig. 2.
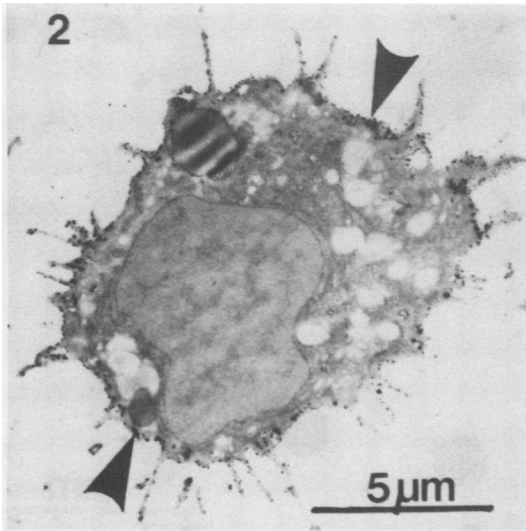
Fig. 2   *Higher power micrograph of a chondrocyte seen in Fig. 1. Note electron dense beads of alkaline phosphatase activity reaction product along the cell membrane (arrows). There is no reaction product inside the cell. (Unstained section.)*

vesicles in disease free, non-osteoarthritic cartilage showed very limited reaction product for alkaline phosphatase compared with the levamisole inhibited controls. Sections of specimens treated with incubation medium containing levamisole, or without an enzyme substrate, showed no reaction product in any region of the cartilage, even around chondrocytes or matrix vesicles less than 40 μm from the nearby tidemark region.

### OSTEOARTHRITIC CARTILAGE

The cartilage examined showed microscopic evidence of surface fibrillation, loss of matrix staining in the superficial region for proteoglycans, and reduced cellularity. We confirmed an increased number of matrix vesicles in the pericellular area in all the cartilage zones but particularly in the deep zone near the tidemark. The tidemark region was usually irregular, and observations by light microscopy indicated its reduplication in several areas. Electron microscopy indicated increased numbers of matrix vesicles and mineral nodules in this region.

In sections incubated for alkaline phosphatase activity there was little evidence of electron dense precipitate around chondrocytes in either the superficial or mid-zone cartilage. In contrast, in all the osteoarthritic specimens studied several chondrocytes in the deep zone (up to 150 μm from the tidemark region) reacted strongly for alkaline phos-

phatase activity (Figs 3–6). In some cases the reaction was strong enough to be observed by light microscopy. In the electron microscope this reaction appeared as an electron dense precipitate along the
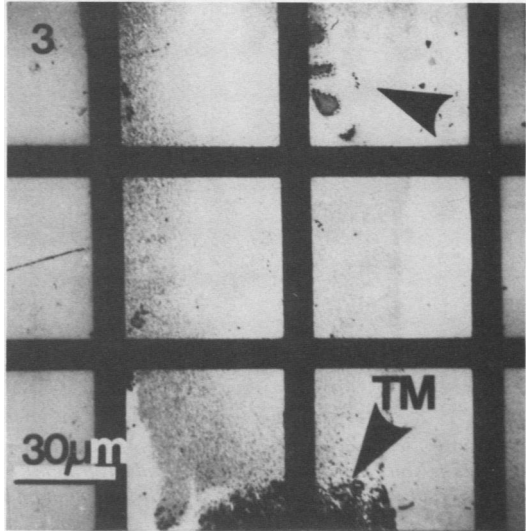


Fig. 3   *Low power electron micrograph of osteoarthritic cartilage incubated for alkaline phosphatase activity (woman, 76 years). Note a cluster of reactive chondrocytes (arrow) about 150 μm from the tidemark (TM). (Unstained section.)*
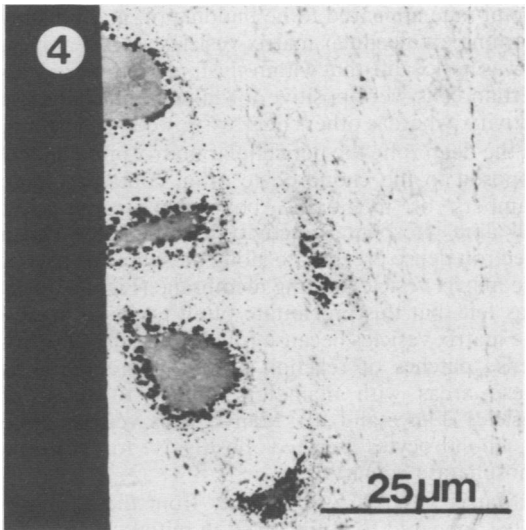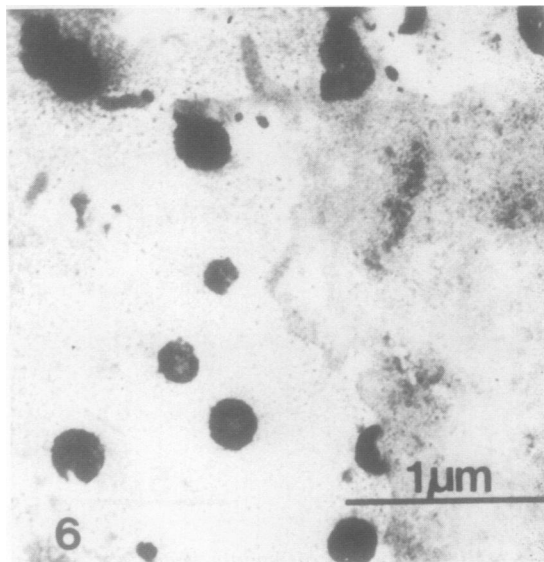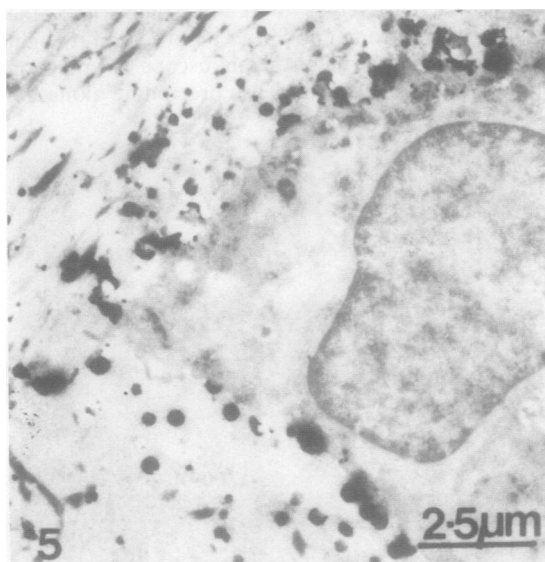


Fig. 4   *Higher power micrograph of the cluster of chondrocytes seen in Fig. 3. Note dense precipitate around the cell membranes and matrix vesicles within the extracellular matrix, positive for alkaline phosphatase activity. (Unstained section.)*

Figs 5 and 6   *Serial section to that seen in Figs 2 and 3. Higher power micrographs of cell membrane of chondrocyte seen in Figs 3 and 4. Note electron dense reaction product on the cell membrane (Fig. 4). There is some evidence that these portions of the cell membrane may be 'budding off' into the surrounding matrix, forming matrix vesicles (Fig. 6). (Stained section.)*

cell membrane of the chondrocytes (Fig. 4). There was considerable variation in the intensity of reaction product found around chondrocytes in the deep zone. In some instances reactive portions of the cell membrane appeared to be budding off and possibly forming extracellular matrix vesicles (Figs 5 and 6). It was noticeable that within clusters of chondrocytes certain cells were positive for alkaline phosphatase activity, whereas others nearby showed no reaction. In the deep zone the pericellular matrix immediately apposed to the chondrocyte often contained large numbers of membrane bound matrix vesicles. Alkaline phosphatase activity was indicated by an electron dense precipitate along the whole or part of the matrix vesicle limiting membrane (Figs 7–10). It was felt that this precipitate often totally obscured the matrix vesicles because roughly circular electron dense patches of reaction product were found in these areas with diameters similar to those of vesicles (Figs 6 and 10). Many matrix vesicles close to chondrocytes reacted positively for alkaline phosphatase activity.

Matrix vesicles further away from the chondrocytes appeared to be involved in initial calcification of the extracellular matrix in the tidemark region by progressive deposition of needle like crystals of hydroxyapatite in and around matrix vesicles. These vesicles showed evidence of alkaline phosphatase activity (Fig. 9). As mineral accumulation appeared

to progress most of the extracellular matrix became calcified and alkaline phosphatase activity reaction product could not be differentiated clearly from calcific deposits.

Control sections, either treated with incubation medium containing levamisole or with the enzyme substrate omitted, showed negligible reactivity for alkaline phosphatase, even around chondrocytes and matrix vesicles in the tidemark region of the cartilage 150 µm from the calcifying front (Fig. 11).

## Discussion

We studied the distribution of alkaline phosphatase activity in human articular cartilage from osteoarthritic joints by an electron microscope technique and compared it with two normal subcapital fracture specimens. Normal cartilage showed little or no reaction in any region, either around chondrocytes or matrix vesicles. In one specimen a cluster of chondrocytes very close to the calcifying front (40 µm away) showed a limited amount of reaction. In contrast, in degenerative cartilage from patients with osteoarthritis of the hip several chondrocytes in the deep zone were always positive for alkaline phosphatase activity. Furthermore, large numbers of matrix vesicles were found within the extracellular matrix, and there is a specific relation between alkaline phosphatase activity, matrix vesicles, and

initial mineral formation in the deep zone as demonstrated by this technique. In this study we were unable to obtain any age matched, normal fresh specimens to provide a direct comparison with the osteoarthritic group. Here we were forced to be content with normal subcapital fracture specimens
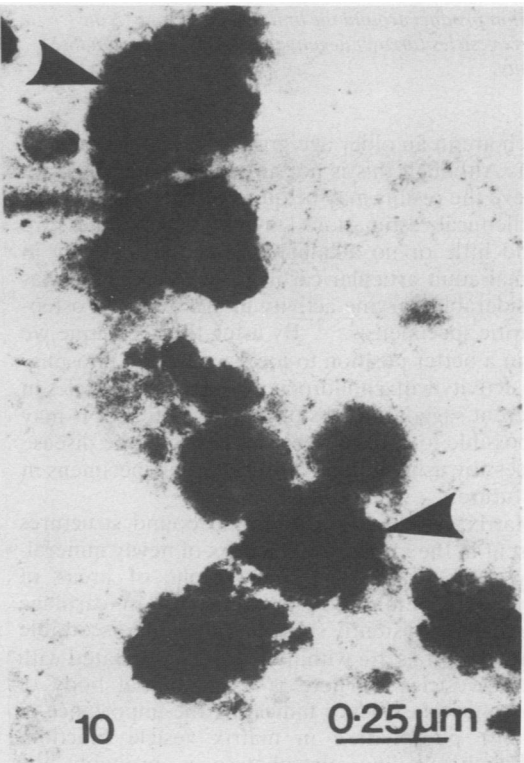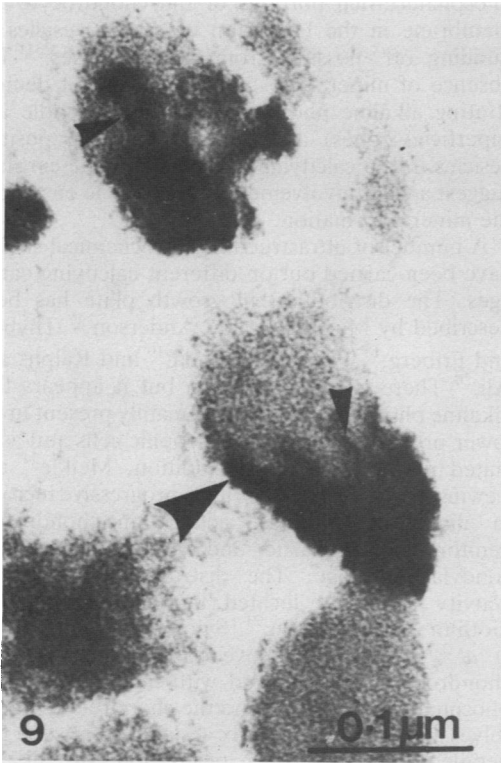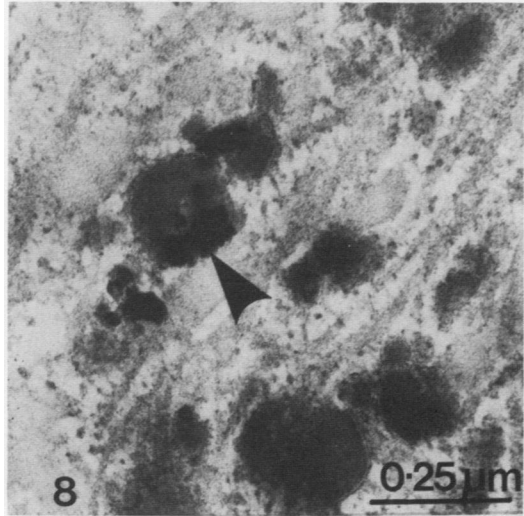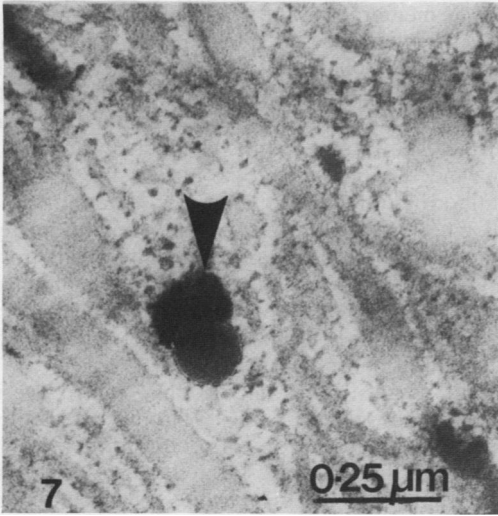


Figs 7–10  *High power micrographs of matrix vesicles in the extracellular matrix in the deep zone of osteoarthritic cartilage incubated for alkaline phosphatase activity. Note alkaline phosphatase activity reaction products (arrows) and initial crystals of hydroxyapatite (small arrows). (Stained sections.)*
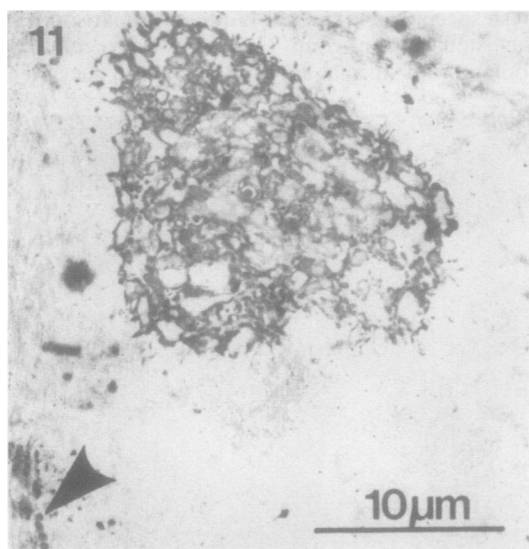
Fig. 11   *Control section (with the specific inhibitor of alkaline phosphatase, levamisole, in the incubation medium). Osteoarthritic articular cartilage (woman, 76 years). A chondrocyte less than 100 μm from the tidemark shows no reaction product around the limiting membrane. Note matrix vesicles (arrow) negative for alkaline phosphatase activity.*

which are in an older age group (83 and 85 years of age). Although this is not an ideal comparison, we believe the results may be quite valid because in our biochemical estimations in previous studies we found little or no alkaline phosphatase activity in normal adult articular cartilage, whereas there was considerable enzyme activity in age matched osteoarthritic specimens.[4 5 27] By using this technique we are in a better position to localise alkaline phosphatase activity with chondrocytes and matrix vesicles in different saggital depths of human cartilage. It may be possible to correlate this activity with the disease process by using a number of different specimens in the future.

Matrix vesicles are membrane bound structures present in the extracellular matrix of newly mineralising tissues. Electron micrographs of areas of incipient mineralisation in a number of cartilage tissues all consistently show that the first discernible mineral crystals are within or closely associated with matrix vesicles.[10] There is a substantial body of experimental evidence indicating the importance of alkaline phosphatase in matrix vesicle function. Although alkaline phosphatase is probably the major enzyme activity in matrix vesicles, its role in initial mineralisation is by no means clear cut and a number of possible roles for this enzyme have been

suggested. The subject of matrix vesicle mediated mineralisation has been reviewed by Ali,[10] Anderson,[28] and Wuthier.[29] Cytochemical and morphological evidence implicates the matrix vesicle membrane as the site of initial mineralisation. This site may represent a logical position to regulate a flux of phosphate ions across matrix vesicles. The lead phosphate reaction product produced by this technique, which is localised at the matrix vesicle surface, may not reflect the complete location of the enzyme.[30] More reliable would be the use of an antibody to recognise antigenic determinants of alkaline phosphatase rather than the present technique which localises enzyme activity. In the future it is hoped to use an electron microscope immunolocalisation technique on sections of articular cartilage to establish further the exact location and function of phosphatase enzymes.

Our results have shown that matrix vesicle alkaline phosphatase activity is only detectable in vesicles in the deep zone of osteoarthritic cartilage, where positive cells are located, suggesting vesicle formation from cell membrane. Evidence obtained in this study (Figs 5 and 6) implicates alkaline phosphatase-rich portions of the chondrocyte cell membrane in the formation of matrix vesicles by budding off, possibly from cell processes.[31] The absence of mineral crystals in vesicles not demonstrating alkaline phosphatase activity (middle and superficial zones) and their presence in positive vesicles in the calcifying deep zone of the cartilage suggest a clear involvement of the vesicle enzyme in the mineral formation.

A number of ultrastructural histochemical studies have been carried out on different calcifying cartilages. The distribution in growth plate has been described by Matsuzawa and Anderson,[17] Thyberg and Friberg,[18] Takagi and Toda,[19] and Ralphs and Ali.[20] There is some variation but it appears that alkaline phosphatase activity is mainly present in the lower proliferative and hypertrophic cells and associated matrix vesicles. In addition, Meikle[24] and Lewinson *et al*[25] have reported a progressive increase in alkaline phosphatase activity in chondroprogenitor, chondroblastic, and hypertrophic cells of condylar cartilage. The distribution of enzyme activity has been located in fracture callus by Gothlin and Ericsson,[21] Salomon,[22] and Volpin *et al*.[23] In all the above cases hypertrophy in chondrocytes is associated with increased alkaline phosphatase activity around the chondrocytes themselves and associated matrix vesicles. This is the first detailed ultrastructural localisation of alkaline phosphatase activity in osteoarthritic human articular cartilage. The presence of increased levels of alkaline phosphatase activity around chondrocytes and matrix

vesicles in the deep zone compared with normal cartilage is consistent with the suggestion that in osteoarthritis articular cartilage (considered by some to be a latent growth plate) has reverted to a growth phase.[4][5][27] This could lead to an advance of the tidemark and subchondral bone. A reduction of the saggital depth of the cartilage or narrowing of the joint space in $x$ ray radiographs may be due to this comparatively rapid mineralisation and advance of the calcification front as suggested by Ali and Evans.[4]

An abnormal advance of the calcification front may make the subchondral region uneven and put greater stress on the remaining cartilage, which may become susceptible to degradation by normal wear processes. Such a pattern over a long period could degrade cartilage in the pressure bearing region.[27][32][33] Changes in local calcium concentration in this area could alter the physicochemical properties of the tissue[34-36] and possibly disrupt the structure of the extracellular matrix as raised levels of calcium in vitro unfold collagen fibrils, and high levels of $CaCl_2$ can extract proteoglycans from cartilage.[37]

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