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Age-related changes in the role of matrix vesicles in the mandibular condylar cartilage *

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INTRODUCTION

The mandibular cartilage of the mouse undergoes marked changes from birth through maturation and ageing. These changes reflect the different developmental requirements imposed upon it. In the young animal it serves as a growth centre for the mandible and is actively involved in endochondral ossification (Koski, 1975; Silbermann & Livne, 1979; Levitan & Silbermann, 1980). The matrix around the hypertrophic cells normally contains extracellular membrane-bound matrix vesicles which display a gradual accumulation of hydroxyapatite crystals, and a positive alkaline phosphatase reactivity, hence contributing to the mineralisation process in this cartilage (Meikle, 1976; Silbermann & Lewinson, 1978). In mature and in old animals the condylar main function is associated with the articulation and mobility of the temporomandibular joint. Marked structural changes accompany this transformation, including a decrease in the tissue cellularity, metachromasia, a relative increase in the collagenous component and an accumulation of matrix vesicles (Livne & Silbermann, 1983). Ultrastructurally, the latter vesicles appear electron-dense and for the most part do not display any microcrystal formation (Livne, von der Mark & Silbermann, 1985). The appearance of such vesicles in regions that are not undergoing mineralisation is of special interest in view of the fact that they are known to serve as an important factor in initiating the mineralisation process (Bonucci, 1967; Anderson, 1969; Ali, 1976; Arsenault & Ottensmeyer, 1983; Dougherty, 1983). Chondrocalcin, a new cartilage matrix calcium-binding protein has been recently characterised (Choi *et al.* 1983) and its distribution in the epiphyseal growth plate cartilage has been demonstrated by the use of monospecific antibodies (Poole *et al.* 1984). In the latter study it was established that chondrocalcin is present in developing non-mineralising cartilage at relatively low intracellular concentrations and that it appears in higher concentration in those extracellular sites that undergo mineralisation.

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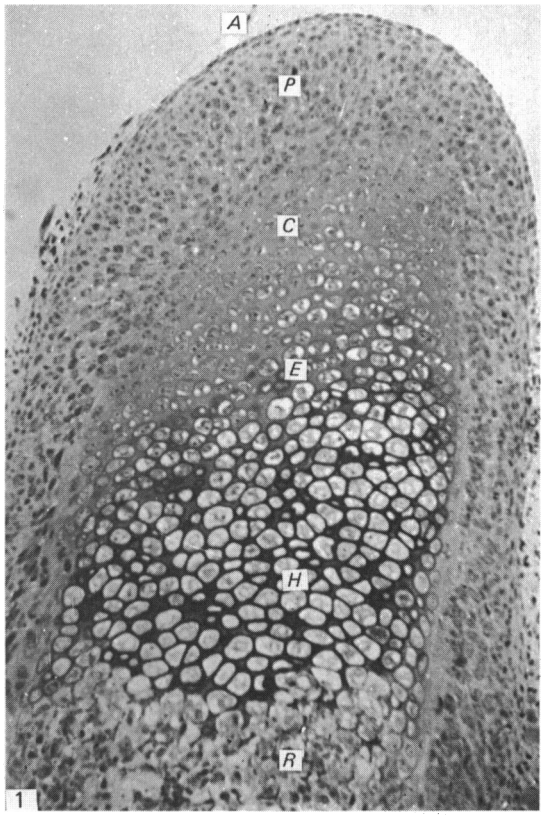

Fig. 1. A frontal section of the mandibular condyle obtained from a one week old animal and stained with toluidine blue. Beneath the articular surface (*A*) one can note a zone of chondroprogenitor cells (*P*), young chondroblasts (*C*), early hypertrophic chondrocytes (*E*), fully matured hypertrophic chondrocytes (*H*) and the resorption front (*R*). ×180.

The present study reports on the localisation of chondrocalcin in the condylar cartilage of young and old animals. In addition, using the electron energy loss spectroscopy method (EELS) (Leapman 1984), an attempt was made to evaluate the calcium content in matrix vesicles at various zones of the condylar cartilage in different age groups. Further, this report reveals that similar vesicles are not associated with the mineralisation of cartilage in ageing animals.

MATERIALS AND METHODS

The mandibular condyles of forty eight CD-1 mice aged from one week to one year were used in this study. The excised specimens were initially fixed in a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0·1 M sodium cacodylate buffer at pH 7·4 for 1–5 hours at 4 °C. Tissues were dehydrated in graded alcohols and embedded in glycol methacrylate (JB-4) resin. One μm-thick sections were stained with 1% toluidine blue for the demonstration of general morphology. For the demonstration of matrix mineralisation at the light microscopy level similar undecalcified sections were stained with the von Kossa stain and counterstained with 0·1% basic fuchsin in 70% ethanol.

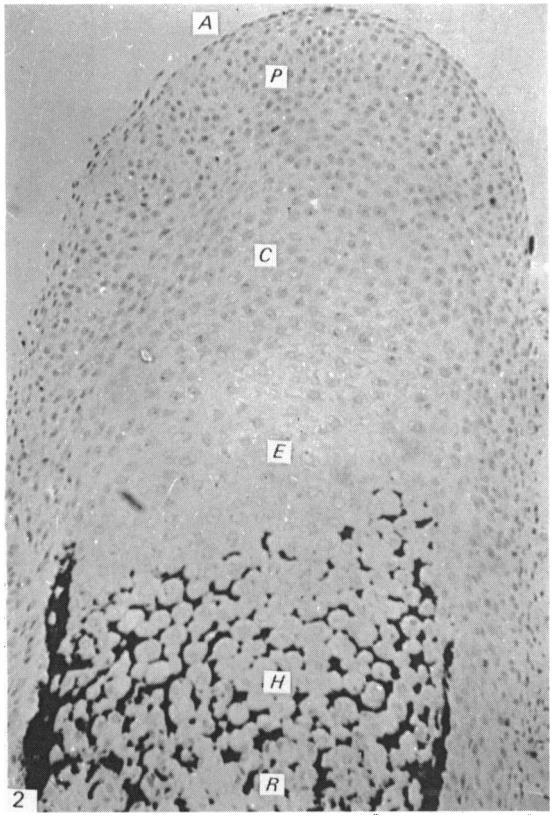

Fig. 2. A similar section to that shown in Figure 1, but stained with the von Kossa stain. Note the heavy mineralisation along the hypertrophic zone (*H*) and the area of primary ossification (*R*). *A*, articular surface; *P*, chondroprogenitor cell zone; *C*, chondroblasts; *E*, early hypertrophic chondrocytes. ×180.

Immunofluorescence microscopy

Frozen sections were attached to microscope slides precoated with 2% gelatin which had been air dried. They were then fixed in 4% formaldehyde for 5 minutes at room temperature, washed for 30 minutes in phosphate-buffered saline, (PBS) and treated with chondroitinase ABC for 1·5 hours at 37 °C. After rinsing in PBS containing 5 mM cysteine for 10 minutes they were treated at room temperature for 30 minutes either with Fab'* from rabbit antiserum R102 to bovine epiphyseal chondrocalcin (with or without preabsorption with chondrocalcin) or with Fab' from pre-immune or non-immune rabbit sera each at a concentration of 3·7 mg/ml in freshly prepared 5 mM cysteine in PBS to produce monovalent Fab'. Subsequent washing and staining of sections was with fluorescein-labelled pig Fab' anti-rabbit Fab' and was followed by fluorescence microscopy as described earlier (Poole *et al.* 1984).

Electron microscopy

Tissue samples for transmission electron microscopy were first fixed in 5% glutaraldehyde in 0·1 M sodium cacodylate buffer (pH = 7·4 at 4 °C) for 90 minutes,

* One of the fragments of the IgG molecule obtained by treatment of the antibody molecule with papain. It contains most of the antigenic determinants.

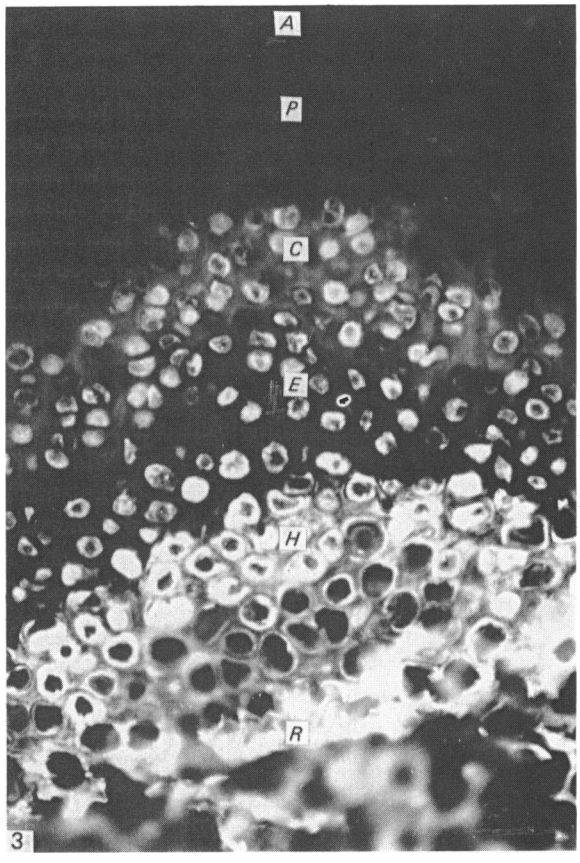

Fig. 3. Frozen undecalcified frontal section from a one week old animal reacted with antibodies against chondrocalcin. Diffuse staining is observed in the young chondroblastic zone (C). At the early hypertrophic cells zone (*E*) the staining appears to be intracellular. Intense immunofluorescence is observed in the matrix around hypertrophic cells (*H*). *A*, articular surface; *P*, progenitor zone; *R*, resorption front. ×200.

postfixed, in 1 % osmium tetroxide in the same buffer (1 hour at room temperature), dehydrated, and embedded in Epon 812 resin. Ultrathin sections (40–50 nm) were cut with a diamond knife on a Porter–Bloom Ultratome, mounted on copper grids and stained with uranyl acetate and lead citrate, and examined in a Zeiss 10A electron microscope. In addition, samples were also fixed and processed without osmication, or were extracted (ethanol, 90 minutes; ethanol : acetone 1 : 1, 90 minutes; acetone, 90 minutes) prior to processing for electron microscopy.

Alkaline phosphatase histochemistry

Tissue slices (500 μm) were fixed in 1·5 % glutaraldehyde in 0·1 M cacodylate buffer (pH 7·2) at 4 °C for 10 minutes. β-glycerophosphate was used as the substrate (Matzuzawa & Anderson, 1971) and, following an incubation for 60 minutes at 37 °C, the tissue was treated with 1 % ammonium sulphide (3 minutes). Specimens were rinsed with the above-mentioned buffer and processed for electron microscopy. Control samples were either incubated in the absence of the substrate or were pre-heated for 60 minutes at 37 °C.

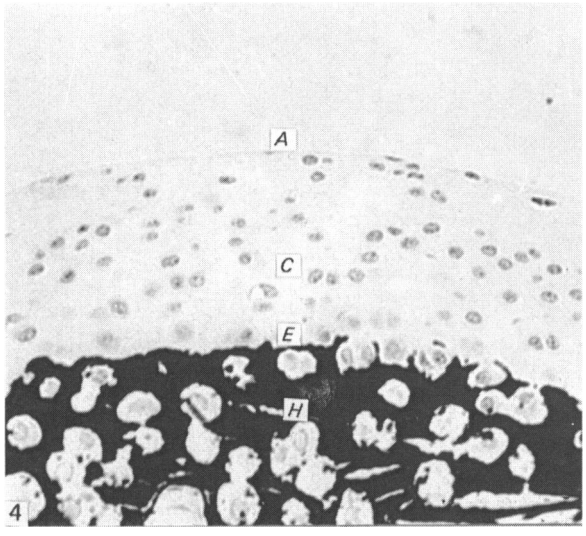

Fig. 4. A frontal section through a portion of the mandibular condyle of a one month old animal, stained with the von Kossa stain. Positive reactivity for matrix mineralisation is noted solely at the lower portion of the condylar cartilage, i.e. along the zone of hypertrophic chondrocytes (*H*). *A*, articular surface; *C*, chondroblasts; *E*, early hypertrophic chondrocytes. ×180.

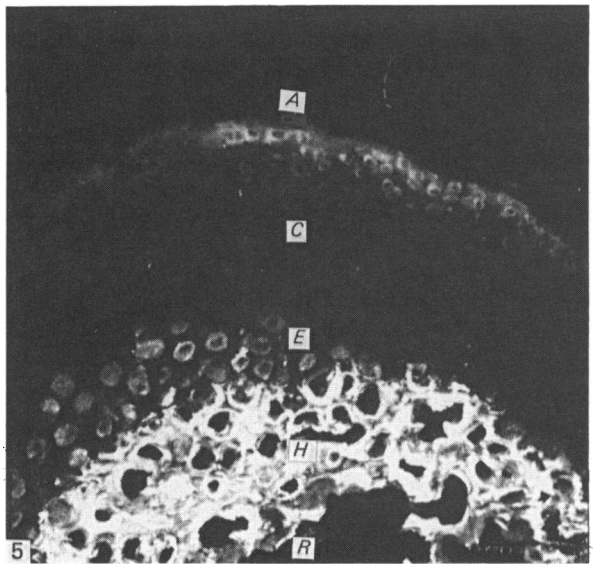

Fig. 5. The pattern of chondrocalcin distribution as seen in one month old condylar cartilage. Intense staining is confined to the matrix of the hypertrophic zone (*H*). Some staining appears intracellularly in the early hypertrophic zone (*E*). The rest of the tissue does not show positive staining for chondrocalcin except for a mild reaction along the articular surface (*A*). *C*, chondroblastic zone; *R*, resorption front. ×200.

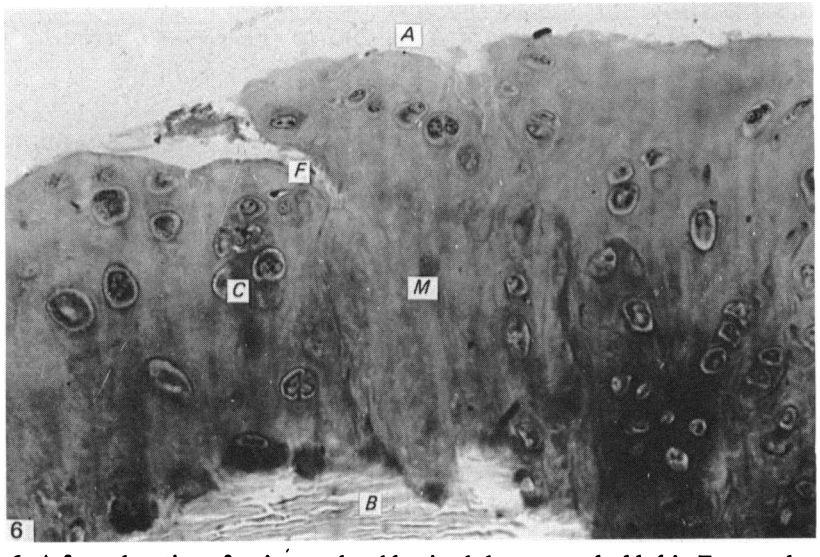

Fig. 6. A frontal section of a six months old animal that was embedded in Epon and stained with toluidine blue. Cleft formation (*F*) is seen along the articular surface (*A*). Note the clusters of chondroblasts (*C*), larger areas of acellular matrix (*M*), and the clear interface between the cartilage and the underlying bone (*B*). ×240.

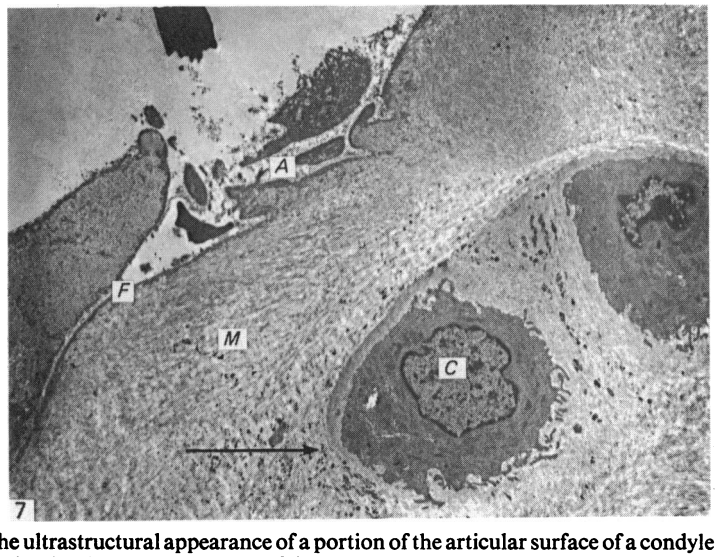

Fig. 7. The ultrastructural appearance of a portion of the articular surface of a condyle of a twelve months old animal. The initial signs of fibrillation (*F*) are seen along the articular surface (*A*). The matrix (*M*) adjacent to a chondrocyte (*C*) contains multiple electron-dense structures (arrow). ×3000.

Electron energy loss spectroscopy (EELS)

Unstained ultrathin (60–70 nm) sections were used for microanalysis of calcium ions. EELS spectra were recorded at 100 keV beam energy using a Hitachi H700H electron microscope equipped with a Hitachi magnetic sector spectrometer. The microscope was operated in the STEM (scanning/transmission electron microscopy) mode with a probe current of 1 nA and a probe diameter of approximately 20 nm. Point

analyses from specific regions were obtained by stopping the probe during a scan, and collecting spectra in a range of from 200 to 700 eV of energy losses. The recorded data were stored in a Digital Equipment Corporation PDP 11/60 computer, where they were analysed.

OBSERVATIONS

The main features of the mandibular condylar tissue are displayed in a frontal section of a one week old animal (Fig. 1). It includes an articular surface, a zone of chondroprogenitor cells, young chondroblasts, early hypertrophic chondrocytes and hypertrophic chondrocytes in the mineralising zone. Underneath the last-named zone an active area of new bone formation is normally noted in neonatal animals. When stained with the von Kossa stain an advanced stage of mineralisation is noticed along the zone of hypertrophic chondrocytes as well as in the primary zone of ossification (Fig. 2).

The distribution of chondrocalcin at this age group is demonstrated in Figure 3. This protein is absent from the articular surface and the chondroprogenitor cells zone, while a relatively diffuse staining is seen in the matrix of chondroblasts. Chondrocalcin reactivity does not appear in the matrix at the early hypertrophic zone where it is confined to the cells themselves. An intense reactivity for this antibody appears in the extracellular matrix around the hypertrophic cells. Controls do not show any positive reactivity.

Marked changes in the tissue architecture are observed in specimens of one month old animals (Fig. 4). At this age group the chondroprogenitor zone is less pronounced and the overall appearance of the tissue resembles that of a typical articular cartilage. The mineralisation is confined to the lower hypertrophic zone, whereas the rest of the tissue is not mineralised. In the same age group the distribution of chondrocalcin is confined mainly to the hypertrophic zone and to a much lesser extent to the articular surface (Fig. 5). Starting at the age of six months the condylar cartilage reveals the initial signs of cartilage disruption (Fig. 6). These defects that usually take the form of clefts and/or ulceration, are occupied by cellular and tissue debris as well as blood cells (Fig. 7). A very characteristic feature of the condylar cartilage of young animals is the appearance of matrix vesicles in the neighbourhood of fully differentiated cartilage cells which are later associated with the mineralisation process (Fig. 8). On approaching the zone of provisional calcification, hydroxyapatite crystals can seen in close proximity to vesicles (Fig. 9). When stained for alkaline phosphatase activity it is evident that both the cellular plasmalemma and the matrix vesicles are the prime sites for the action of this enzyme (Fig. 10).

A completely different feature is noted in condylar cartilage of older (one year old) animals. In these cases large accumulations of structures resembling matrix vesicles are consistently noted adjacent to chondrocytes at various regions of the non-mineralised portions of the tissue. These structures appear as electron-dense vesicles that are embedded amongst collagen fibres and do not reveal any signs of crystal formation (Fig. 11). The electron density of these vesicular structures is maintained even after the extraction procedure by which the lipid material was removed from the tissue (Fig. 12).

Further substantiation of the above observations was obtained through the use of the EELS approach. Figure 13 represents a spectrum that was recorded from that area of the cartilage lacking mineralisation, and it can easily be seen that no calcium

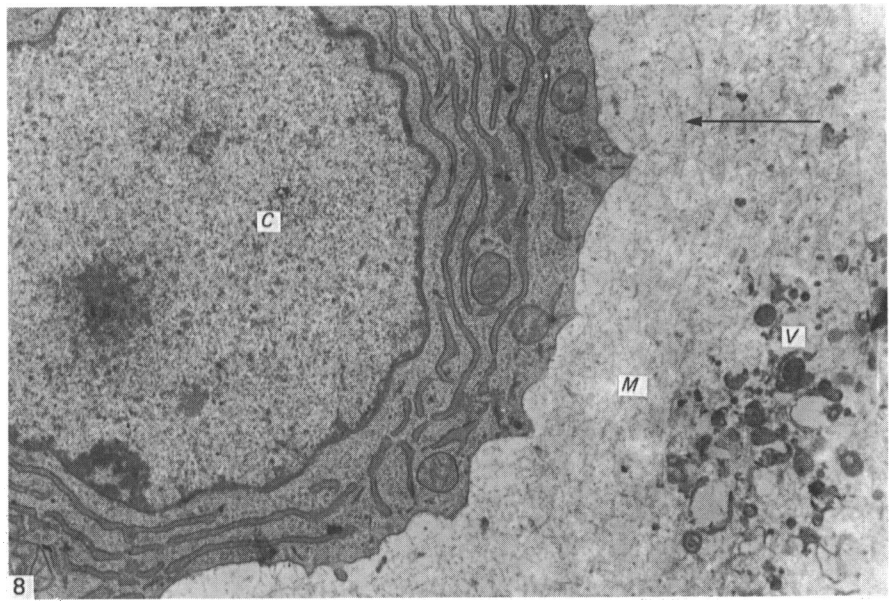

Fig. 8. A portion of an early hypertrophic chondrocyte (*C*) in the condylar cartilage of a one week old animal. The matrix (*M*) adjacent to this cell reveals a cluster of membrane-bound matrix vesicles (*V*). Some of these vesicles appear empty and some contain electron-dense material. Very fine collagen fibrils (arrow) are in the matrix. ×16000.

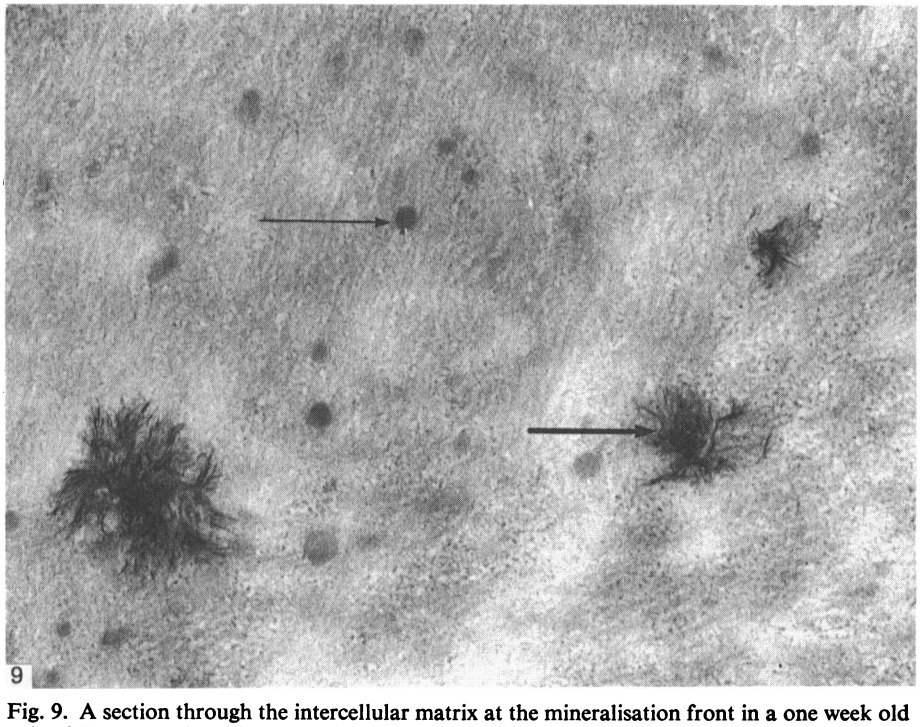

Fig. 9. A section through the intercellular matrix at the mineralisation front in a one week old animal. Note matrix vesicles (thin arrow) and the hydroxyapatite crystals (thick arrow) associated with them. ×40000.

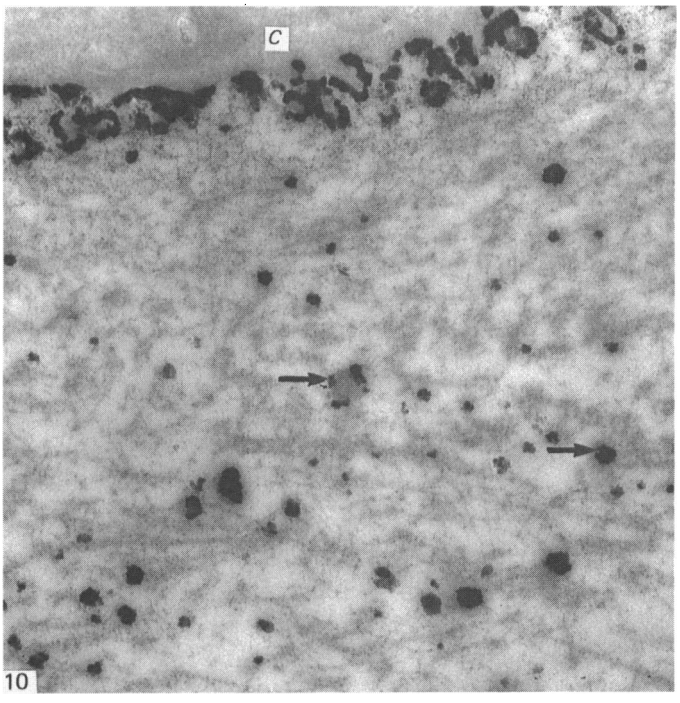

Fig. 10. The appearance of condylar cartilage that has been treated for alkaline phosphatase, using the Matzuzawa & Anderson method. Note the intense reactivity along the chondroblastic (*C*) plasmalemma as well as in the matrix vesicles (arrows). × 30000.

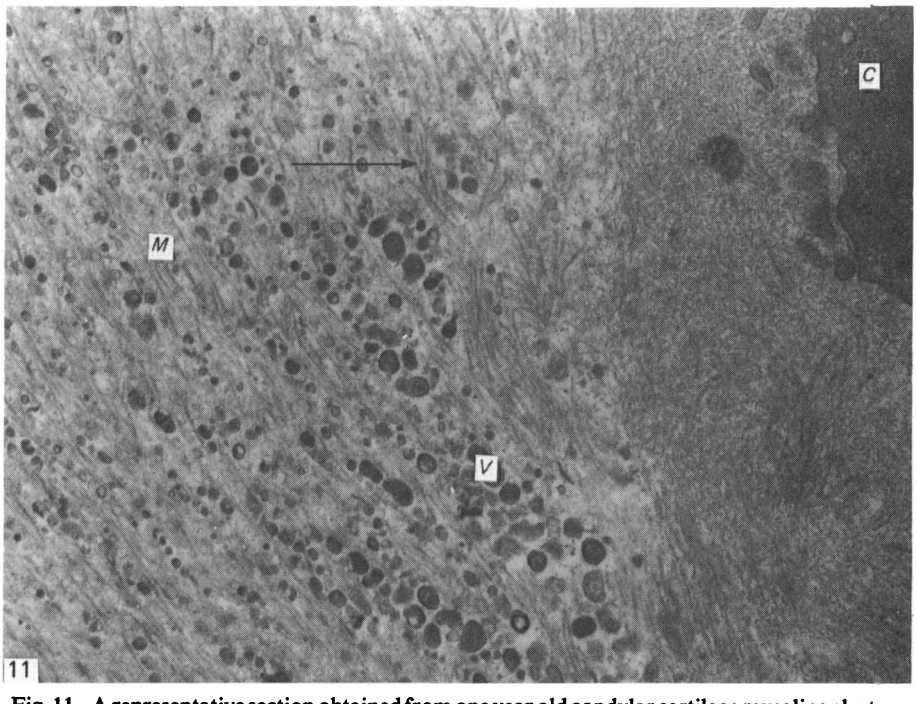

Fig. 11. A representative section obtained from one year old condylar cartilage revealing electron-dense matrix vesicles (*V*) embedded within the matrix (*M*) adjacent to a chondroblast (*C*). Note multiple thick collagen fibres in this area (arrow). × 25200.

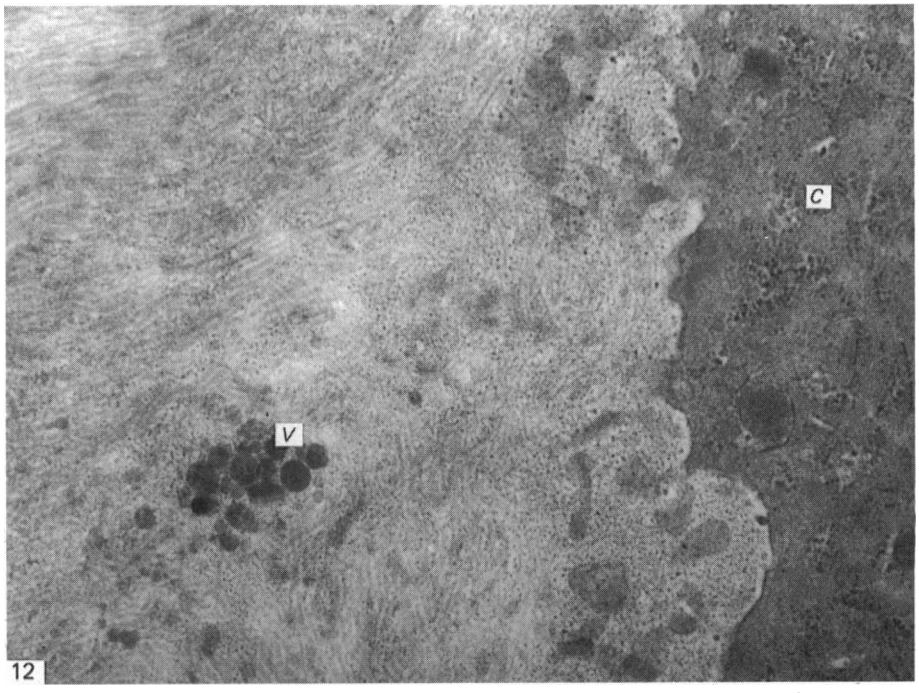

Fig. 12. A section through a portion of chondrocyte (*C*) and its adjacent matrix following an extraction procedure as described in Materials and Methods. Note the persistence of the electron-dense characteristics of the existing matrix vesicles (*V*). ×30000.

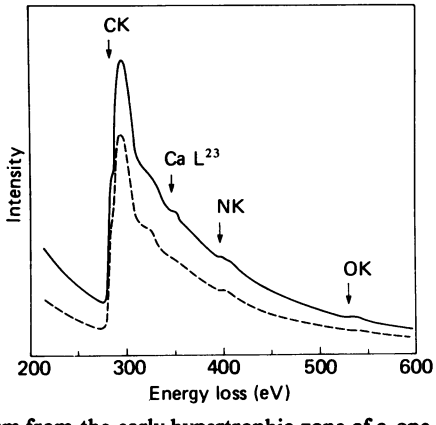

Fig. 13. EELS spectrum from the early hypertrophic zone of a one year old animal. Note the shallow shoulder in the calcium region along the spectral curve. Solid line, spectra recorded inside vesicles. Broken line, spectra recorded at the adjacent matrix. CK, carbon edge; NK, nitrogen edge; OK, oxygen edge; Ca L_{23}, calcium edge.

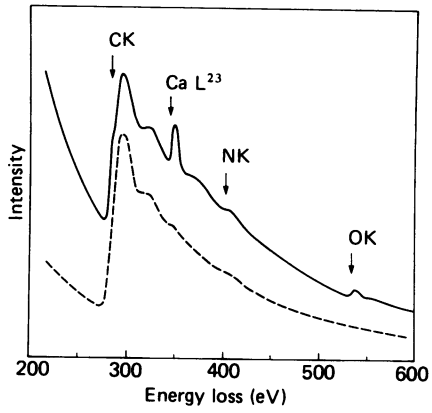

Fig. 14. A similar EELS spectrum as shown in Figure 13, but obtained from confined region in the mineralised zone. Note the prominent peak of calcium as depicted within matrix vesicles (solid line).

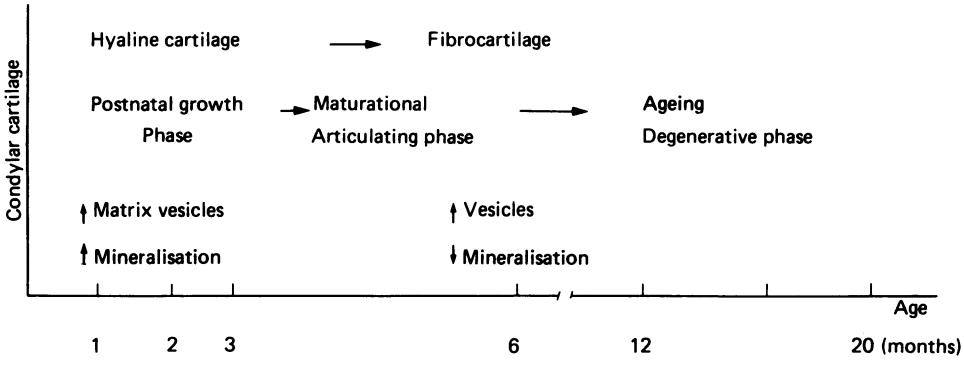

Fig. 15. A schematic representation of the age-related changes taking place in the mandibular condylar cartilage of mice.

atoms are depicted. Using the same approach for regions in the mineralised hypertrophic zone, does indeed reveal discrete peaks for calcium ions within mineralising matrix vesicles (Fig. 14).

DISCUSSION

In the present study we examined the changes that take place in mandibular condylar cartilage of mice in different age groups. Our findings indicate towards a topographical correlation between the distribution of chondrocalcin and von Kossa positive reactivity. Marked morphological changes were observed as the animal ages. In this study we have reiterated the role of matrix vesicles in cartilage mineralisation as has been previously described in young animals and in other mineralising tissues such as bone, dentine and cartilage (Bonucci, 1967; Anderson, 1969; Ali, 1976; Dougherty, 1978). These membrane-bound structures are considered to be involved in the initiation of mineralisation and once the initial foci of apatite have been established, further mineralisation seems to progress by secondary crystallisation from previously formed crystals.

Chondrocalcin, the cartilage matrix calcium-binding protein that has been purified from fetal epiphyseal cartilage, was found to bind strongly to hydroxyapatite and to be intimately associated with the major mineralisation sites of cartilage matrix

(Choi *et al.* 1983; Poole *et al.* 1984). A similar pattern of chondrocalcin distribution was found in the condylar cartilage where the most intense staining occurred in the mineralising hypertrophic zone. The fact that we noticed staining for chondrocalcin in and around chondroblasts of young animals may indicate that this molecule also appears very early in chondroblastic expression. In the condylar cartilage, as in the epiphyseal growth plate, the chondroprogenitor cells did not contain or secrete detectable amounts of chondrocalcin. Moreover, we have previously shown that such weaker staining for chondrocalcin is observed in association with cells and matrix of the developing bovine epiphysis in sites where mineralisation does not occur (Poole *et al.* 1984). The significance and function of chondrocalcin in these chondroblastic sites remain to be established.

Various techniques have been used to assist the conventional microscopic imaging of the dynamic events of mineralisation, among them the potassium pyroantimonate staining (Appleton & Morris, 1979), ultracryomicrotomy for increased preservation of matrix ground substance (Landis & Glimcher, 1982) and the use of specific antibodies to localise both the monomer core protein and the link protein of proteoglycans (Poole, Pidoux & Rosenberg, 1982). Electron energy loss spectroscopy (EELS), the technique used in the present study provides a reliable approach for the determination of calcium content in mineralising tissue. The advantage of this method is that the spectrum originates only from those atoms sampled by the electron probe. It is assumed that the embedding material replaces water in the sample preparation; the molar concentration of calcium in the embedded section corresponds roughly to that in the tissue. Within the limitations of such technique, it became apparent that in both young and old animals the highest contents of calcium occurred in vesicles located in areas where apatite crystals could be identified ultrastructurally and were positive for von Kossa.

One of the features characterising the condylar cartilage of old animals is the fact that it contains increasing amounts of matrix vesicles in areas that are not undergoing mineralisation. Similar membrane-bound structures have been found in other ageing cartilages (Meachim, 1969; Dearden, Bonucci & Guicchio, 1974). It has been suggested that they are involved in the lysis of chondrocytes and that their number increases when the articular surface is damaged (Mitchell & Shepard, 1970). The lack of spatial correlation between the localisation of matrix vesicles and that of mineral deposits, as noted in articular cartilage of our old animals, indicates a possible shift in the role of matrix vesicles in cartilage metabolism and turnover. This shift coincides with the gradual change from the typical hyaline-type cartilage that characterises the condyle in young animals to a fibrocartilage usually noted in ageing animals (Fig. 15). Consequently the structures that resemble matrix vesicles are not consistent with the classical notion of matrix vesicles and may represent a different population of membrane-bound structures (Y. S. Ali, personal communication), engaged in different metabolic activities that reflect the new environmental needs of the maturing temporomandibular joint.

SUMMARY

A combined approach of light microscopy, immunofluorescence, transmission electron microscopy and electron energy loss spectroscopy (EELS) was used to study age-related changes in the condylar cartilage in mice. Chondrocalcin, a cartilage matrix calcium-binding protein, was demonstrated by indirect immunofluorescence

microscopy using monospecific antibodies. In one week old animals the most intense staining was observed in the matrix around the hypertrophic cells in the mineralising zone, to a lesser degree around the cells in the zone of chondroblasts, while no staining was noted in the zone of chondroprogenitor cells and in the matrix around the early hypertrophic cells. In the mineralisation zone the distribution of chondrocalcin correlated with that of mineral deposits as revealed by the von Kossa stain. The matrix between the early hypertrophic cells as shown by transmission electron microscopy revealed the presence of matrix vesicles and demonstrated a gradual accumulation of hydroxyapatite in the mineralising zone. In one month old animals chondrocalcin localisation was mainly confined to the lower hypertrophic zone which also demonstrated positive von Kossa staining was seen along the articular surface.

In older animals multiple electron-dense structure that resembled matrix vesicles were observed in the non-mineralising portions of the condylar cartilage. Use of the EELS method confirmed the almost complete lack of calcium ions in these structures. In contrast, with the use of the same method, detectable amounts of calcium were recorded in vesicles in the mineralising zones of all age groups. Hence what appear ultrastructurally as structures similar to matrix vesicles represent atypical vesicles that might characterise an ageing and degenerative articular cartilage and are not necessarily associated with the mineralisation process.

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