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The fine structure of developing elastic cartilage

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INTRODUCTION

Although a number of detailed studies of cartilage have been made at the ultrastructural level, most of these have concentrated on hyaline, particularly hyaline articular, cartilage (for reviews see Meachim & Stockwell, 1973; Stockwell & Meachim, 1973), much less attention being given to elastic cartilage. One of the earliest papers on elastic cartilage was that of Sheldon & Robinson (1958): they examined rabbit ear cartilage and were able to identify a material that they considered to be elastica. They later studied the effects of the injection of papain on the cartilage (Sheldon & Robinson, 1960; Sheldon & Kimball, 1962). Subsequently, Anderson (1964) compared cartilage from the trachea and femur with ear cartilage in the rat. Recently, Serafini-Fracassini & Smith (1974) have described young rat and adult bovine ear cartilage in the course of a review of the structure and biochemistry of cartilage in general.

The structure of the elastic cartilage of the developing rabbit ear varies with age and precise location. The cartilage never becomes uniform in appearance: morphological gradients are always present. Consequently, a fully comprehensive account needs to consider position as well as time. The present paper describes the changes that occur at set intervals along the midline of the rabbit ear from base to tip and the more marked changes that supervene with age. Inevitably, many subtle relationships will have been missed. However, various stages in the morphogenesis of elastic cartilage have been demonstrated and a necessary qualitative background provided for more detailed quantitative investigations. The presence of different zones of development in hyaline articular and epiphysial cartilage is well documented but, as far as can be ascertained, little has been written on elastic cartilage in this respect. The opportunity has also been taken to compare the ultrastructure of developing elastic cartilage with that of hyaline cartilage as described by other authors.

MATERIALS AND METHODS

The ears of New Zealand white rabbits aged 1, 3, 6, 10, 15, 20, 28, 36, 49, 59, 73, 87, 116, 140, 186, 744 and 1108 days were selected as the source of elastic cartilage. The pinna was measured from its attachment to the skull to its tip, and marks made at 1 cm intervals along the midline. The animals were killed and small blocks of cartilage were taken from the various marked regions of the pinna. As the pinna did not always measure an integral number of cms, blocks were routinely taken from

the tip region. The tissue was immediately fixed at 4 °C in 2 % glutaraldehyde (Sabatini, Bensch &Barrnett, 1963) in Millonig's phosphate buffer (Millonig, 1961) at pH 7·4 for 2½ hours, washed overnight in buffer and post-fixed in 2 % osmium tetroxide, also in Millonig's phosphate buffer, at pH 7·4 and at 4 °C, for 2 hours. The blocks were dehydrated in graded alcohols and embedded in Araldite (Luft, 1961). Thin sections were cut on either an LKB Ultratome or a Porter-Blum MT2 microtome, mounted on collodion-coated grids, and positively stained with uranyl acetate followed by lead citrate (Echlin, 1964). Many additional pieces of tissue were block-stained with 2 % uranyl acetate in distilled water for 17 hours at 60 °C (Locke, Krishnan & McMahon, 1971) and the sections positively stained with lead citrate (Reynolds, 1963). This method of block-staining was found to extract glycogen, but in doing so often it revealed other details of structure. The sections were examined in an A.E.I. E.M. 6B2 electron microscope that had been calibrated using a carbon replica of a diffraction grating and catalase crystals (Cox & Horne, 1968). Extensive use was made of photographic montages.

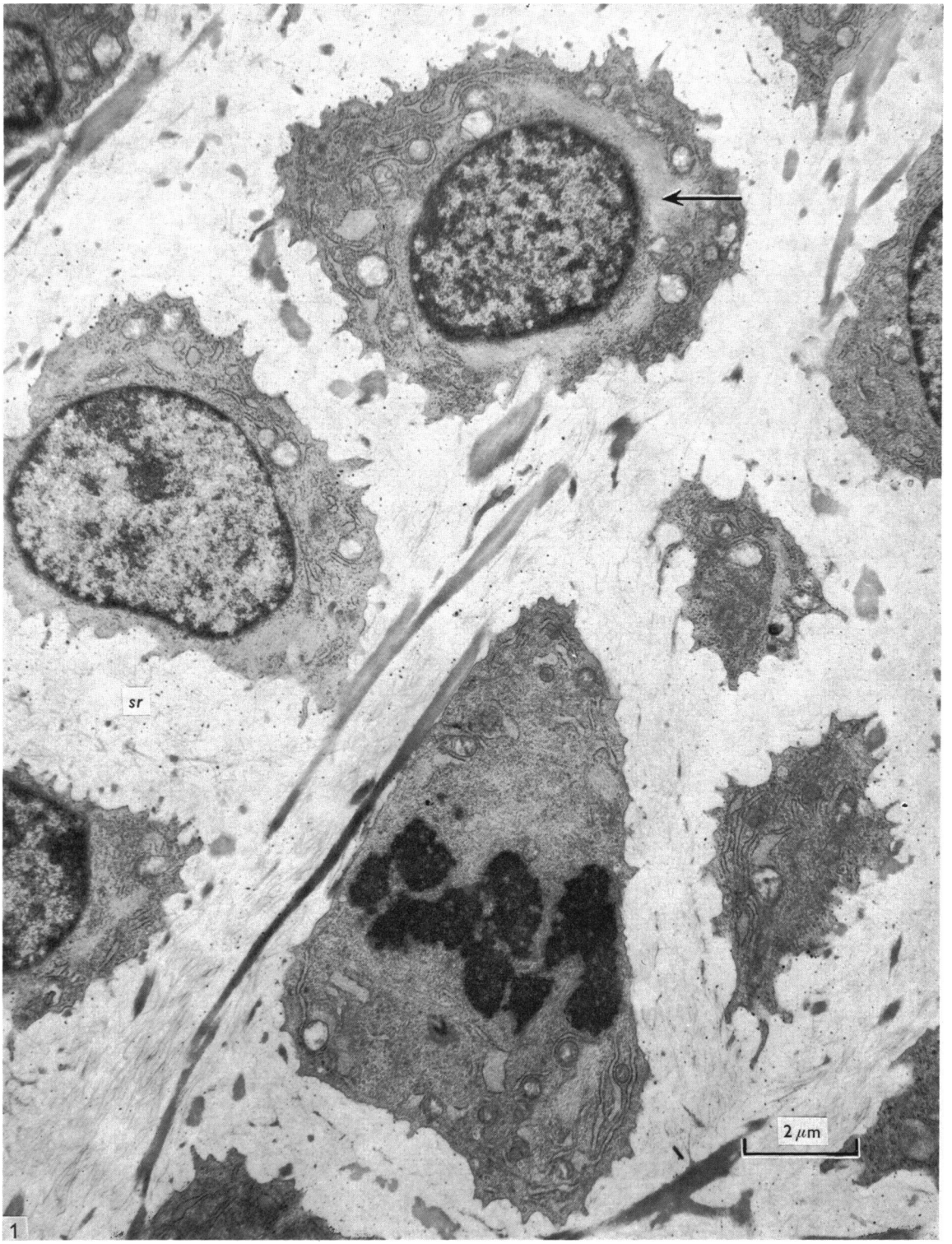
RESULTS

Chondroblasts

Until 15 days the nuclei at the tip of the ear are circular or oval in shape with a nuclear envelope which, apart from nuclear pores, is regular and relatively free from indentations. This shape is preserved throughout most of the length of the ear, but at the base the nuclear envelope of occasional cells is wavy or indented. By 49 days, however, irregularity of the nuclei has progressed the whole length of the ear and even those at the tip may show prominent indentations. It is difficult to detect an unequivocal change in the size of the nuclei from tip to base in any single ear and of the nuclei of cells at corresponding sites of ears of different ages. Until approximately 20 days there is a tendency for the chromatin to concentrate round the nuclear envelope and for aggregates to occur within the nucleoplasm. Thereafter the chromatin is evenly distributed. A nucleolus with nucleolonema is distinguishable in a number of cells, and cells with two nuclei are occasionally seen in all situations from the tip to the base. Even at 1108 days a nucleolus may be present and cells with two nuclei may be observed. Mitotic figures are seen frequently at 1, 3 and 6 days (Fig. 1) and sometimes thereafter until 20 days.

The cells themselves appear approximately round or oval in shape, but closer inspection reveals an extremely irregular surface with innumerable fine, branched, cytoplasmic processes extending into the intercellular substance. The effect is to increase enormously the surface area of the cells. The impression is given that this irregularity of the cell surface increases from the tip of the ear to the base and that

Fig. 1. Elastic cartilage situated 2 cm from the base of the ear of a 6 day old rabbit. One cell shows a mitotic figure but in others chromatin is present as aggregates within the nucleus. Cytoplasmic microfilaments produce the light areas seen in the cells (arrow). Rough endoplasmic reticulum and cytoplasmic processes are evident, but the latter become more prominent later on. Elastica, some of it in apposition with the cell surface, is conspicuous. Dense particles of stellate reticulum are seen between the cells (*sr*). Staining on the grid with uranyl acetate and lead citrate has been used for all figures except 5 and 6 where block-staining was employed.


sr
2 μm
1

there is some variation with the age of the particular ear studied. Thus the processes appear to increase in number (Figs. 1 and 3) from 1 day onwards and are still very prominent at 1108 days. Plasmalemmal vesicles (Fig. 2) or coated vesicles are present and are sometimes situated in the cytoplasmic processes. The plasmalemmal vesicles are distinguishable from 1 day onwards, but are less prominent after 49 days, although even at 1108 days some can still be detected. There is a progressive and striking increase in cell size from 1 to 140 days, when enlargement ceases. This increase in size is associated with the accumulation of a great deal of intracellular lipid (Fig. 6) after about the 36th day. Lipid droplets are clearly discernible even at 1 day, but the quantity of lipid increases very slowly until about 28 days. The lipid is distributed in increasing amounts from the tip to the base. By the 140th day many cells at the base of the ear consist almost entirely of lipid. Even at this stage, however, there is still an increase in intracellular lipid from the tip to the base, suggesting a permanent gradient. The lipid droplets do not possess a distinct unit membrane.

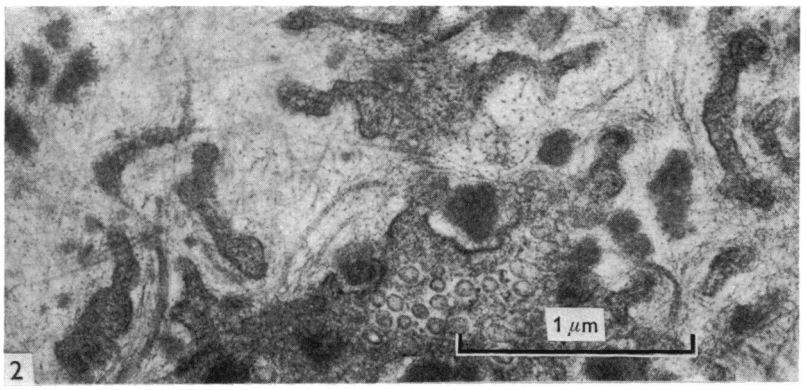
Solitary cilia (Fig. 4) and centrioles are visible at 1 day, and are common by 15 days, but become progressively less frequent thereafter. The solitary cilia are distinguishable until 36 days and the centrioles until 73 days. In the early stages solitary cilia and centrioles are noted at all levels of the ear. After 15 days the cilia occur more towards the tip, but the centrioles still appear at all levels. Contiguous cells are encountered, and occasional desmosomes are demonstrable at 1 day.

Rough endoplasmic reticulum (Fig. 1) and ribosomes free in the cytoplasm are prominent at 1 day, but are much less so after 36 days. They are present at all levels of the ear, but vary from cell to cell. Dilatation of the cisternae of the rough endoplasmic reticulum is more apparent at the base of the ear, the dilated cisternae containing granular material. This dilatation is perceptible even at 1 day, but again varies with the individual cell. Smooth endoplasmic reticulum and mitochondria are recognizable in most cells, the latter being less frequent towards the base. Both types of structure are seen at all ages, but are progressively less obvious after 49 days. An occasional large mitochondrion measuring at least 35 μm in length is observable. Golgi apparatus is prominent until 73 days, but even at 744 days can still be distinguished. The large vesicles of the Golgi complex, however, are infrequent in the early stages, but are more evident from 36 to 49 days. Residual bodies containing myelin figures are detectable from 1 to 36 days. Lysosome-like bodies are themselves visible at all times until 1108 days, at which time the amount of lipid leaves little cytoplasm to examine. They occur at all levels of the ear, as do occasional unclassifiable vacuoles. Cytoplasmic microfilaments (Figs. 1, 5 and 6) are also discernible at all levels of the ear, either near the nucleus or just inside the periphery of the cell.

Fig. 2. Plasmalemmal vesicles in a chondroblast from a site 3 cm from the base of the ear of a 744 day old rabbit.

Fig. 3. Portion of a chondroblast from the tip of the ear of a 28 day old rabbit. Cytoplasmic processes are more conspicuous than at 6 days. Some of these partly surround pieces of elastica (arrow). Mitochondria are present within the chondroblast and stellate reticulum is again evident in the intercellular matrix.

Fig. 4. A single cilium in a chondroblast of the elastic cartilage of a 6 day old rabbit. The site is near the tip of the ear.



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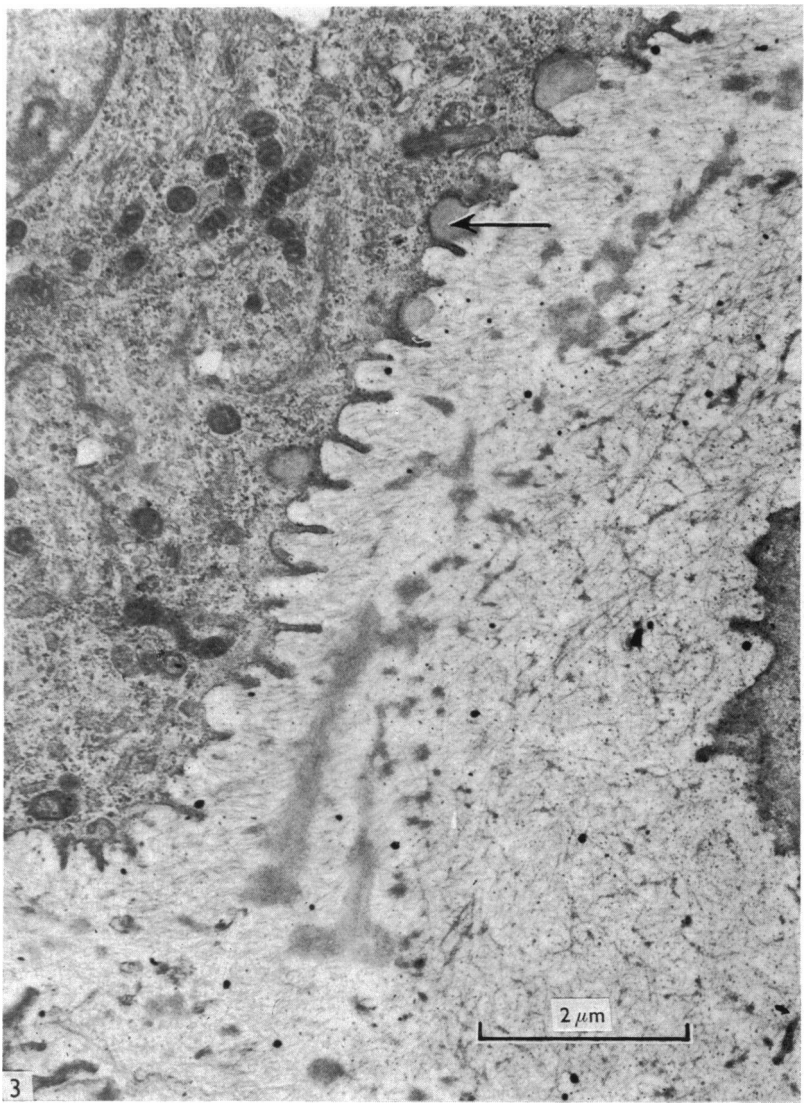

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The microfilaments are present at 1 day and increase with age, being very distinct at 140 days when cell enlargement has ceased. They are particularly well seen round lipid droplets. By 1108 days microfilaments, along with lipid droplets and nucleus, fill most of the cell, few other organelles being present.

Glycogen is seen at 1 day. It is present as discrete particles which increase in number from the tip to the base. By 15 days large aggregates have formed at the base and by 36 days these extend to the tip. Glycogen increases with age until approximately 186 days, when it is overshadowed by the amount of lipid present.

Occasional cells are seen with dense cytoplasm and nucleus and markedly dilated endoplasmic reticulum containing granular material. These cells are similar to those described in hyaline articular cartilage by Palfrey & Davies (1966) and classified by them, on morphological grounds, as degenerate cells.

Matrix

The amount of intercellular material apparently increases with age, and from the tip to the base of the ear, the cells becoming more widely separated. The immediately pericellular tissue looks relatively immature, with few fibrils present. Further from the cell actual collagen fibrils are recognizable; these increase in thickness and become more prominent as sections from the tip to the base are examined, but show little attempt at orientation. Similarly, with age, the collagen fibrils of the intercellular matrix increase in number and thickness, but again exhibit little orientation.

Elastica (Fig. 1), which is visible at 1 day, is present at all levels of the ear. At the tip many fragments are in apparent apposition with the plasma membrane, some being partly surrounded by the cytoplasmic processes already mentioned (Fig. 3). Formation of elastica is usually associated with a part only of the plasma membrane of the cell at any time. Towards the base of the ear most of the elastica is situated further from the cell. The elastica tends to lie further away from the plasmalemma of the cell with time (Fig. 6) and coalesces into irregular networks round the cells. The strands of this network are eventually situated about midway between adjacent cells, and every cell appears to be separated from its neighbours by a partial invest ment of elastica. The elastica is formed mainly of amorphous material, but an associated microfibrillar component of approximately 10 nm in diameter can some-- times be distinguished. With uranyl acetate and lead citrate applied to the sections on the grid, the staining of the elastica becomes progressively less intense with time. From approximately 36 days onwards the elastica stains poorly. However, the use of block-staining with 2% uranyl acetate and subsequent positive staining of the sections with lead citrate renders the elastica electron-dense on all occasions.

Matrix vesicles are present in the intercellular matrix (Fig. 8) at 1 day: they increase with age and are very prominent at 140 days, 186 days and 1108 days. They are seen at all levels of the ear, but tend to be more numerous at the tip. On occasions matrix vesicles seem to be released into the intercellular material from vacuoles at

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Fig. 5. The periphery of a chondroblast in the cartilage of a 140 day old rabbit. A vacuole full of matrix vesicles is releasing its contents into the intercellular matrix. Elastica is seen near the cell margin and cytoplasmic microfilaments and lipid are situated within the cell.


2 μm
5

the periphery of the cell (Figs. 5 and 6). Such vacuoles are conspicuous from 36 days onwards. The matrix vesicles (Fig. 7) vary considerably in diameter, although most measure 100–200 nm. Many possess a trilaminar membrane, but others are multilaminar. Needle-like crystals are not discernible, but electron-dense material is frequently present in the centre of the vesicles. Circular or oval vesicles are common, but spirals and elongated structures are also prominent. In addition to matrix vesicles, small dense particles (Figs. 1 and 3), stated by Smith (1970) to be a stellate reticulum composed of non-collagenous protein, are seen from 1 day to 1108 days at all levels of the ear.

The above description is of cartilage situated approximately midway between the dorsal and ventral aspects of the ear. As the cartilage approaches the perichondrium the cells become flattened and rather disc-shaped. Actual bundles of collagen fibrils appear and these become thicker and more dense as the perichondrium is entered. The cells of the perichondrium are flattened or elongated with dense bundles of collagen fibrils running in different directions between them. Some elastica is still present. The perichondrium represents the boundary of the cartilage, and is considered to be its main source of chondroblasts during development. The cell volume fraction of this boundary layer always remains less than unity.

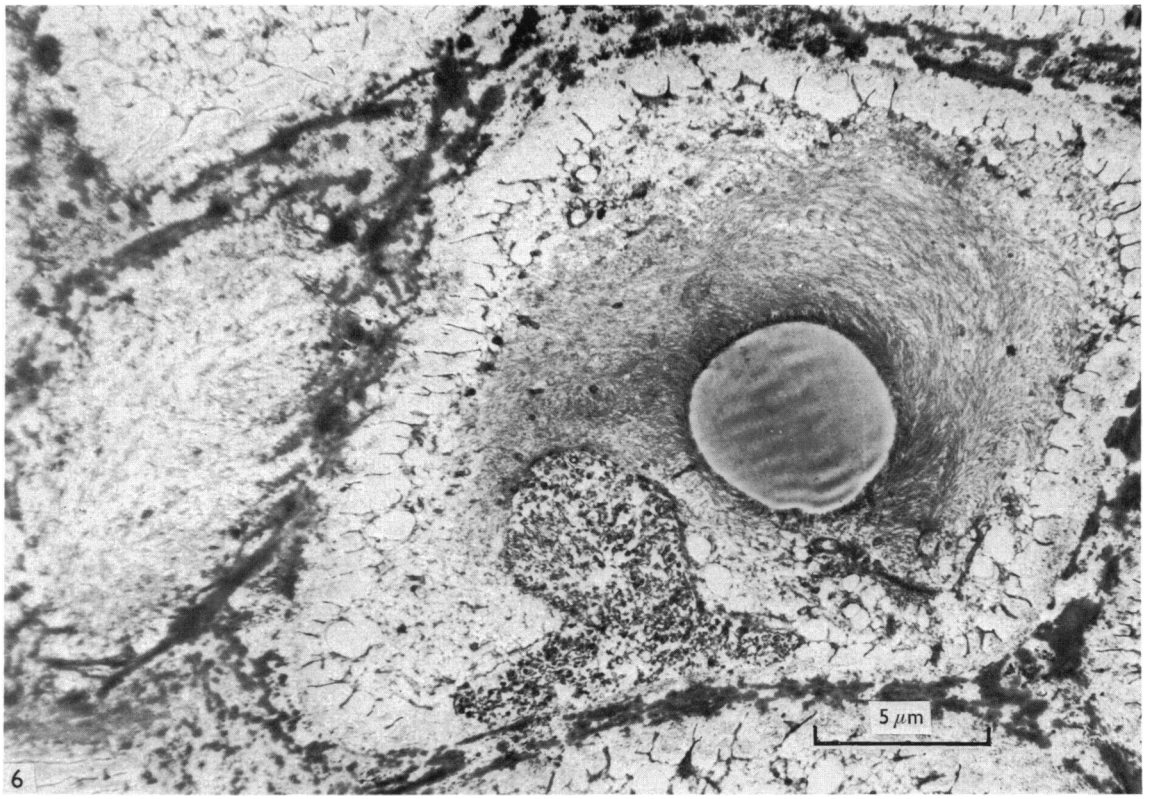
DISCUSSION

Examinations of sections taken at regular intervals from the midline of the ear suggests that progressive maturation of the cartilage is occurring from the tip to the base. These alterations in the cartilage with position must be allowed for when considering changes associated with time. Thus, it appears that the quantity of intercellular material increases from the tip to the base, and also increases with time. Furthermore, there is a striking increase in cell size with time. While it is possible that the intercellular volume fraction also increases from the tip to the base at a given time it is not obvious what happens to the intercellular volume fraction at a given site as time increases. Similarly, the impression is gained that the amount of irregularity of the surface of individual cells increases from tip to base. However, bearing in mind the increase in intercellular material, it is difficult to assess the variation in cell surface area per unit volume of tissue from tip to base at any given time or, at a given site, the variation with time. Many of these variations can only be assessed quantitatively.

Fig. 6. Matrix vesicles being released into the intercellular matrix from a vacuole in a chondroblast. The cell contains many cytoplasmic microfilaments which surround the lipid droplet present. Cytoplasmic processes are seen at the edges of the cell and an investment of elastic is prominent. Elastic cartilage situated 11 cm from the base of the ear in a 140 day old rabbit.

Fig. 7. Details of matrix vesicles seen in the ear cartilage of a 744 day old rabbit at a site 3 cm from the base. There is considerable variation in diameter. Electron-dense material is present within some matrix vesicles but no needle-like crystals are visible. Some vesicles are multilaminar.

Fig. 8. Elastic cartilage showing matrix vesicles in an intercellular position. Elastica and stellate reticulum particles are also visible. The situation is 3 cm from the base of the ear of a 36 day old rabbit.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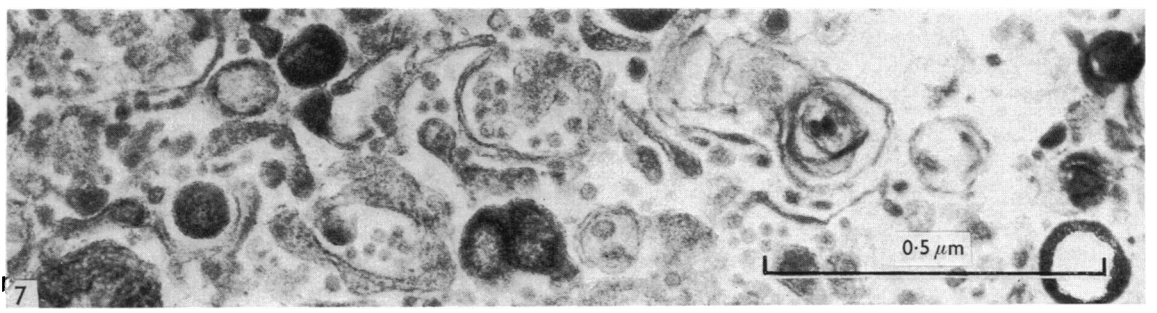
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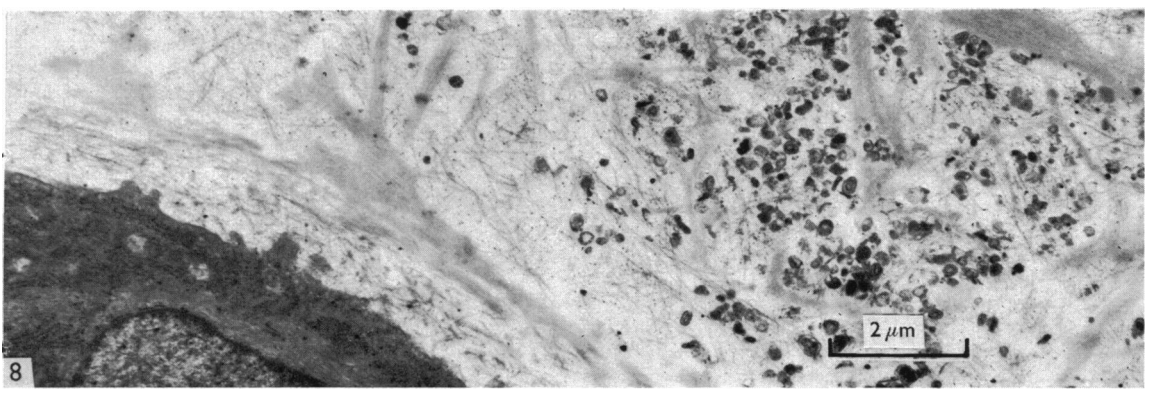
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Whether the plasmalemmal vesicles observed are true pinocytotic vesicles, or are evidence of secretory activity in the opposite direction, or both, is difficult to judge. In articular cartilage the vesicles are termed micropinocytotic, implying that they transport material into the cell from the exterior (Stockwell & Meachim, 1973). However, exocytosis is also to be inferred from the known role of chondroblasts in the formation of ground substance and collagen (Meachim & Stockwell, 1973), and from the intimate association of small portions of elastica with the plasma membrane in the present investigations (Fig. 3) and earlier work (Anderson, 1964). Although formation of elastica occurs at an earlier age than the extensive accumulation of lipid in the cartilage cells, it is of interest that surface vesicles similar to the plasmalemmal vesicles already described were noted and examined by Cushman (1970*a*, *b*) in adipose cells of the epididymal fat pads of rats. A proportion only of these vesicles was shown, by the uptake of gold, to be pinocytotic and Cushman suggested that an exocytic system might also exist.

Serafini-Fracassini & Smith (1974) have commented on the paucity of large Golgi vesicles in the chondrocytes of the auricular elastic cartilage of young rats when these cells are compared with chondrocytes in other tissues, such as rabbit epiphysial plate. This finding is also true of the rabbit elastic cartilage of the present study. Since Palfrey & Davies (1966) have reported desmosomes between the cells of articular cartilage and Smith, Christie & Frame (1969) between those of epiphysial cartilage, the occurrence of desmosomes between the chondroblasts of the elastic cartilage of the rabbit ear is probably to be expected.

A single cilium with two centrioles has now been observed in a number of tissues that are not normally ciliated (Wheatley, 1967; Milhaud & Pappus, 1968). Single cilia have been reported in mouse (Scherft & Daems, 1967), guinea-pig (Hart, 1968) and young adult human articular cartilage (Stockwell & Meachim, 1973) and in rabbit auricular cartilage (Smith *et al.* 1969). The significance of a single cilium in these cells has not been elucidated and the frequent observation of centrioles is no easier to explain (Fulton, 1971). The function of the cytoplasmic microfilaments in the chondroblasts remains obscure although their presence in many other cells has been associated with contractile processes (Rutter, Pictet & Morris, 1973). In the rabbit ear cartilage the microfilaments are present at 1 day but increase with age. In bovine auricular cartilage Serafini-Fracassini & Smith (1974) saw cytoplasmic filaments in mature cartilage only, and not in younger specimens. In chondroblasts of the rabbit ear the microfilaments eventually tend to form a capsule round the lipid droplets, and with the latter to replace most of the other cell organelles.

The appearance and behaviour of lipid droplets in cartilage cells is intriguing, but has received little detailed attention. Stockwell & Meachim (1973) state that most workers are agreed on the non-degenerative nature of the intracellular lipid found as globules in the chondrocytes of articular cartilage. The amount of lipid in elastic cartilage increases markedly with age and eventually replaces most of the cell at 1108 days. At first sight, the lipid would seem to be a convenient store of chemical energy. However, whether this lipid acts merely as a depot that can be metabolized, or has other functions, is still uncertain.

The glycogen in hyaline cartilage is thought to provide a store of chemical raw material, possibly partly for the synthesis of glycosaminoglycan (Stockwell &

Meachim, 1973). This accumulation of glycogen is very marked in some cells of the rabbit ear elastic cartilage, however, In other chondroblasts the infiltration is much less prominent and is overshadowed, in time, by the lipid accumulation. It is possible that in the rabbit the glycogen is involved in the synthesis of these triglycerides.

Silberberg & Silberberg (1961) state that there is scanty knowledge regarding age changes in elastic cartilage, and that there is no apparent ontogenetic or phylogenetic consistency in the age at which elastic fibres appear. In this connexion it is of interest that Sacerdotti (1900) did not observe elastica before the 15th day post partum in rabbit ear cartilage. However, elastica was seen at birth in the present study, although the amount increased rapidly from then onwards. In fact, even with the light microscope, tinctorial elastica may be seen in the proximal half of the rabbit ear cartilage at 26 days of fetal age, and has almost reached the tip by 31 days of fetal age.

Anderson (1964) described the elastica in the cartilage of the ear of the infant rat (14–16 g). At this stage the elastic consisted of irregular clumps and strands of dense homogeneous material that sometimes touched the cell. Although there were no definite ultrastructural features, occasional suggestions of longitudinal fibrillar elements and periodic transverse banding were seen.

Greenlee, Ross & Hartman (1966), Greenlee & Ross (1967) and Ross & Bornstein (1969) have examined ligamentum nuchae in fetal calves and flexor digital tendons in fetal and young rats. They reported two different constituents of the mature elastic fibre, a central amorphous and a peripheral microfibrillar component. The microfibrils stained well with cationic lead and uranyl acetate and the amorphous component with anionic phosphotungstic acid. The microfibrils appeared before the amorphous material, were approximately 10 nm in diameter, and were usually orientated in parallel array.

Serafini-Fracassini & Smith (1974) found that in mature tissues such as adult bovine auricular cartilage the elastic fibres stained lightly with cationic stains, and appeared to be entirely amorphous, with no indication of a microfibrillar component. In young rats they noted that the matrix contained large fibres which were amorphous, but differed from the mature tissue in that they stained intensely with cationic stains. Where these fibres ran out of the plane of section the dense amorphous material appeared to continue for a short distance as microfibrils. They thought that the amorphous appearance might be due to such a close packing of the longitudinal microfibrils that their individuality was obscured, except when present in very thin layers.

In the current study of the rabbit ear cartilage microfibrils of approximately 10 nm were seen associated with the amorphous component, although the latter was the more prominent. Microfibrils that could be unequivocally labelled elastic tissue were always associated with the amorphous component. However, this is not altogether inconsistent with the findings of Greenlee, Ross, Hartman & Bornstein in their various papers, as the rabbit cartilage described in the present paper is not fetal but post-term. These authors emphasized that the microfibrils were formed in close approximation to the cell surface and were often found in niches or infoldings of the cell. The amorphous component which represented the protein elastin would aggregate between and round the microfibrils. The amorphous material increased with fetal age, so that by term the elastic fibres consisted largely of this component.

To resolve some of the points raised by Serafini-Fracassini & Smith would possibly require the examination of fetal rather than post-term elastic cartilage.

The term 'matrix vesicles' is apparently used by Anderson (1973) to include both the matrix vesicles of types I and II of Thyberg & Friberg (1972) and both the matrix dense bodies and matrix vesicles of other authors (Serafini-Fracassini & Smith, 1974). The usage of Anderson is adopted in the present paper. The origin of matrix vesicles in calcifying cartilage has been a subject of speculation (Bonucci, 1967, 1970; Anderson, 1969, 1973; Thyberg & Friberg, 1970, 1972). In the present experiments numerous structures were seen that appear to be similar to those described by Barnett, Cochrane & Palfrey (1963) and Ghadially, Meachim & Collins (1965) in hyaline cartilage and, except for needle-like crystals, by Anderson (1973) in calcifying cartilage and bone matrix. While these matrix vesicles in elastic cartilage are certainly frequently observed in the intercellular matrix near the plasmalemma of cells, the intracellular aggregation of similar vesicles into cytoplasmic vacuoles, whose contents are then apparently freed into the intercellular matrix, has been a much more arresting phenomenon. This aggregation is particularly well demonstrated in cells that have large lipid droplets. Within the small amount of non-lipid cytoplasm of these cells there are a few small structures that would seem to be either electron-dense mitochondria, lysosomes or remnants of endoplasmic reticulum. Some of these structures bear considerable resemblance to the vesicles in the vacuoles. The impression is gained that the vesicles are derived partly from cell organelles and partly from plasmalemma and are collected into vacuoles at the periphery of the cell before being released into the intercellular tissue. In elastic cartilage the presence of matrix vesicles does not appear to be associated with calcification.

SUMMARY

The fine structure of the elastic cartilage of the pinna has been examined in young rabbits aged from 1 day to 1108 days. Changes associated with growth and development are related not only to age but also to the actual situation in the pinna. In the midline, progressive changes are seen from the tip to the base. The changes in the chondroblasts with time are compared with those described in hyaline cartilage. Structures occur that, except for the presence of crystals, are apparently morphologically identical with the matrix vesicles of calcifying cartilage. These matrix vesicles, however, become very prominent with age, and aggregations of them appear to be released into the intercellular tissue from vacuoles at the periphery of the chondroblasts. There is no obvious association with calcification. Occasional single cilia, desmosomes and giant mitochondria are seen. Elastica is present at birth, and eventually every cell is separated from its neighbours by a partial investment of elastica. The quantity of matrix seems to increase with time, and with distance from the tip of the ear. This is accompanied by a marked increase in cell size with time.

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