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An Ultrastructural Study on the Ear Cartilage of Rabbits After the Administration of Papain

Appearance of Cross-Striated Collagen Segments of an Atypical FLS-Type

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Summary. Crude papain was administered intravenously to young rabbits and the cartilage of the collapsed ear was examined electron-microscopically. Degeneration and recovery of chondrocytes, and decrease in and recovery of the electron-density of elastic fibers, were observed during the collapse and restoration of the ear. Some samples were stained with ruthenium red. In the collapsed ear, with a marked decrease of proteoglycan in the cartilage, loss of ruthenium red-positive granules was observed in the extracellular matrix. Collagen fibrils in the cartilage appeared to be somewhat increased in number, some of their diameters became slightly greater, and a part were assembled into bundles, occasionally accompanied by periodic cross-striation. Decrease of proteoglycan in the cartilage matrix probably brought about the unmasking and the assembly of collagen fibrils. In one of the experimental animals, collagen fibrous segments of an atypical fibrous long spacing (FLS-)type with symmetrical cross-striation were found around the chondrocytes in the ear cartilage, during the period of recovery. Some kind of the endogenous sulfated carbohydrate may have acted to affect the arrangement of type II collagen or procollagen molecules newly produced by the recovering chondrocytes.

Key words: Ear cartilage – Papain – Collagen fibril – Elastic fiber – Atypical FLS segments

Introduction

Proteoglycans and collagen are the main extracellular components of cartilage. Recent reports have shown that collagen aggregation and fibrillogenesis are controlled by proteoglycans and glycosaminoglycans in vitro (Öbrink 1973; Gelman and Blackwell 1974; Oegema et al. 1975; Franzblau et al. 1976; Ander-

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son et al. 1977) and probably in vivo (Myers et al. 1973; Trelstad et al. 1974; Borcherding et al. 1975). In an attempt to examine ultrastructual changes in the distribution, organization and size of collagen fibrils in the cartilage by the alteration of its extracellular matrix, we observed the ear cartilage of young rabbits injected with a solution of crude papain. Intravenous administration of crude papain to young rabbits results in collapse of the ears (Thomas 1956), accompanied by biochemical changes of proteoglycans in the extracellular matrix of the ear cartilage (Tsaltas 1958). A similar effect is not produced by injection of crystalline papain because it readily reacts with its substrate in the circulating blood and does not reach the ear, whereas papain inactivated by a thiol-combining agent such as iodo-acetate can get to the ear cartilage where it is converted to its active form (McClusky and Thomas 1958). Crude papain is thought to be protected from interaction with its substrate in the blood and was thus used in the present experiment.

Sheldon and Robinson (1960) first made electron microscopic observations on the cartilage of the collapsed ears of rabbits given papain. However, the preparation of specimens (especially embedding) was different from that now generally employed and their results were not all consistent with those obtained in the present study. In the course of this study, we found cross-striated fibrous segments of an atypical FLS-type, which were produced by collagen aggregation. This paper is concerned with cross-striated segments of this unusual type as well as the ultrastructural changes in cells and extracellular components in the ear cartilage of rabbits given crude papain. In several specimens, we used ruthenium red for staining of the matrix and tannic acid for staining of the elastic component.

Materials and Methods

White young rabbits, weighing about 1 kg, were used. Ten ml of 1% solution of crude papain (Merck, co.) in physiological saline was administered intravenously once, to 25 rabbits. Specimens of the ear cartilage were obtained 2, 4, 8, 12, 18, 24, 48, 72 and 96 h later. Small pieces, of the specimens about 1 mm^3, were fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) at 4° C for 1 h, dehydrated in graded ethanols and embedded in Epon 812. Ultrathin sections were prepared on a Reichert OmU2 ultramicrotome, stained with uranyl acetate and lead citrate, and examined under Akashi TRS-80 and S-500 electron microscopes. Several specimens were fixed in 0.5% cetyl pyridinium chloride in 4% neutral formaldehyde solution, embedded in paraffin wax, and sections were stained with toluidin blue at pH 2.8.

Staining with Ruthenium Red. Some specimens from normal and papain-treated rabbits were used for staining of proteoglycan with ruthenium red, according to the method of Shirahama and Cohen (1972). The tissue blocks were cut into small pieces (approximately $2 \times 2 \times 1$ mm) with a razor blade, and immersed in 2% paraformaldehyde-2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 1,000 ppm ruthenium red at 4° C for 2 h. The tissues were then minced into further smaller pieces (approximately $1 \times 1 \times 0.5$ mm), and fixed in the same aldehyde fixative containing ruthenium red for an additional 2 h. The specimens were washed briefly in 0.1 M cacodylate buffer with 1,000 ppm ruthenium red, and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) containing the same concentration of ruthenium red at 4° C for 1 h. After rinsing in the cacodylate buffer, they were dehydrated in ethanol, embedded in Epon, and prepared for electron microscopy. Ultrathin sections from the ruthenium red-treated specimens were stained with only uranyl acetate.

Staining with Tannic Acid. Some sections were stained with tannic acid, according to the method of Kajikawa et al. (1975). Tannic acid, 0.3 g, and 0.5 g of paranitrophenol were dissolved in 25 ml of distilled water by heating. After cooling 0.7 ml of 5% uranyl acetate was added to this solution. The final solution was applied for staining of sections for 10 min. After rinsing in distilled water, these sections were stained with lead citrate for 5 min.

Results

Ear Cartilage of Normal Rabbits

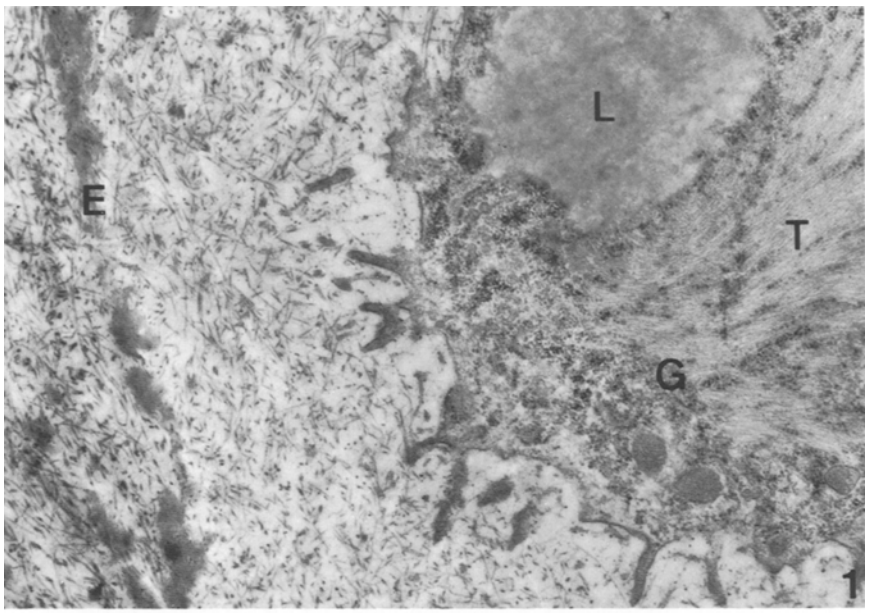
a) Chondrocytes. The ultrastructure of the chondrocytes in the rabbit ear cartilage was not uniform even in the normal state. Most cells contained a small number of lipid spherules, many small mitochondria, moderately developed rough-surfaced endoplasmic reticulum, Golgi apparatus, abundant tonofilaments and some glycogen granules (Fig. 1). Some cells were filled with so many clusters of glycogen granules that various cell organelles were hardly visible (Fig. 2). Both types of chondrocytes had varying amounts of cell organelles, but the surface of all cells examined looked serrated, with many projections of the cytoplasm.

b) Extracellular Matrix. The extracellular space was filled with a delicate mesh work of thin collagen fibrils, 140–200 Å in diameter, without periodical cross-striation. Elastic fibers of a dense, amorphous appearance lay nearly midway between chondrocytes (Fig. 1). Light-microscopically the extracellular matrix was well-stained metachromatically with toluidine blue (Fig. 3), and electron-microscopically proteoglycan of the matrix could be seen with ruthenium red-staining, as small electron-dense granules of irregular shapes largely attached to collagen fibrils (Fig. 5).

Ear Cartilage of Rabbits Treated with Papain

Intravenous injection of a solution of crude papain to young rabbits brought about a complete collapse of both ears within 24 h, and 3 or 4 days afterwards the ears gradually returned to their initial form.

a) Chondrocytes. Two to 18 h after the administration of papain, at the light microscope level chondrocytes of the ear became large, round and close together. This change was accompanied by loss of basophilia in the extracellular matrix and reduction in the amount of matrix (Fig. 4). Electron-microscopically, many cells showed marked swelling with considerable cytoplasmic oedema and a diminution of intracellular organelles and glycogen granules. The initially indented cell surface changed into a rather smooth one (Figs. 8, 9). In some cells, in contrast, the cytoplasm looked rather shrunken and remarkably vacuolated and the cell membrane was more serrated with fine cytoplasmic processes (Fig. 7). Over 18 h after the papain treatment, many chondrocytes began to show signs of recovery, and about 24 h later they came to contain well-developed,



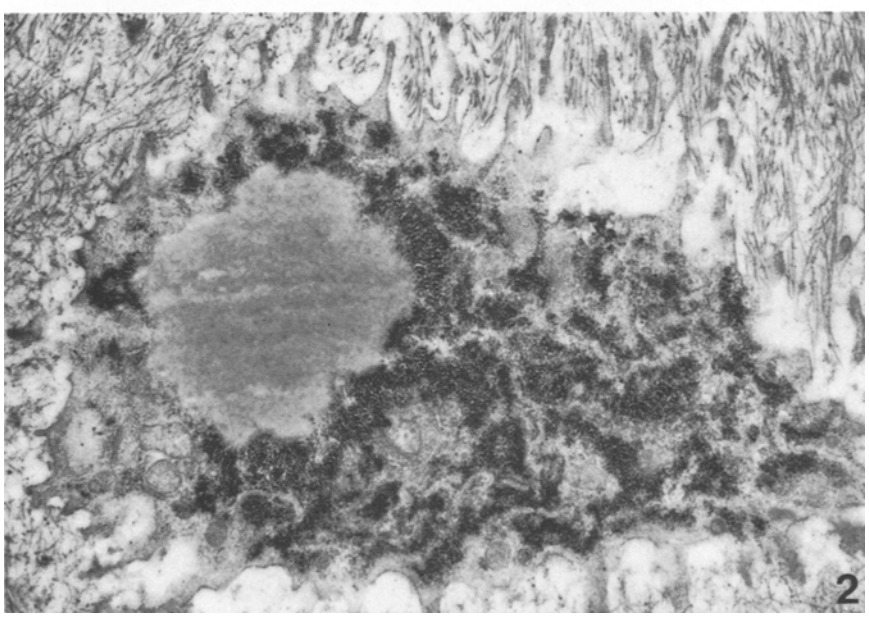


Fig. 1. Normal rabbit ear cartilage. A chondrocyte shows a markedly indented cell surface. The cytoplasm contains abundant tonofilaments (*T*), a lipid spherule (*L*) and some glycogen granules (*G*). In the extracellular space there are numerous fine collagen fibrils, about 120–150 Å in diameter, without cross-striation. Elastic fibers (*E*) are seen at some distance from the cell. ×18,000

Fig. 2. Normal rabbit ear cartilage. This chondrocyte is characterized by a large number of glycogen granules distributed in the cytoplasm. ×18,000

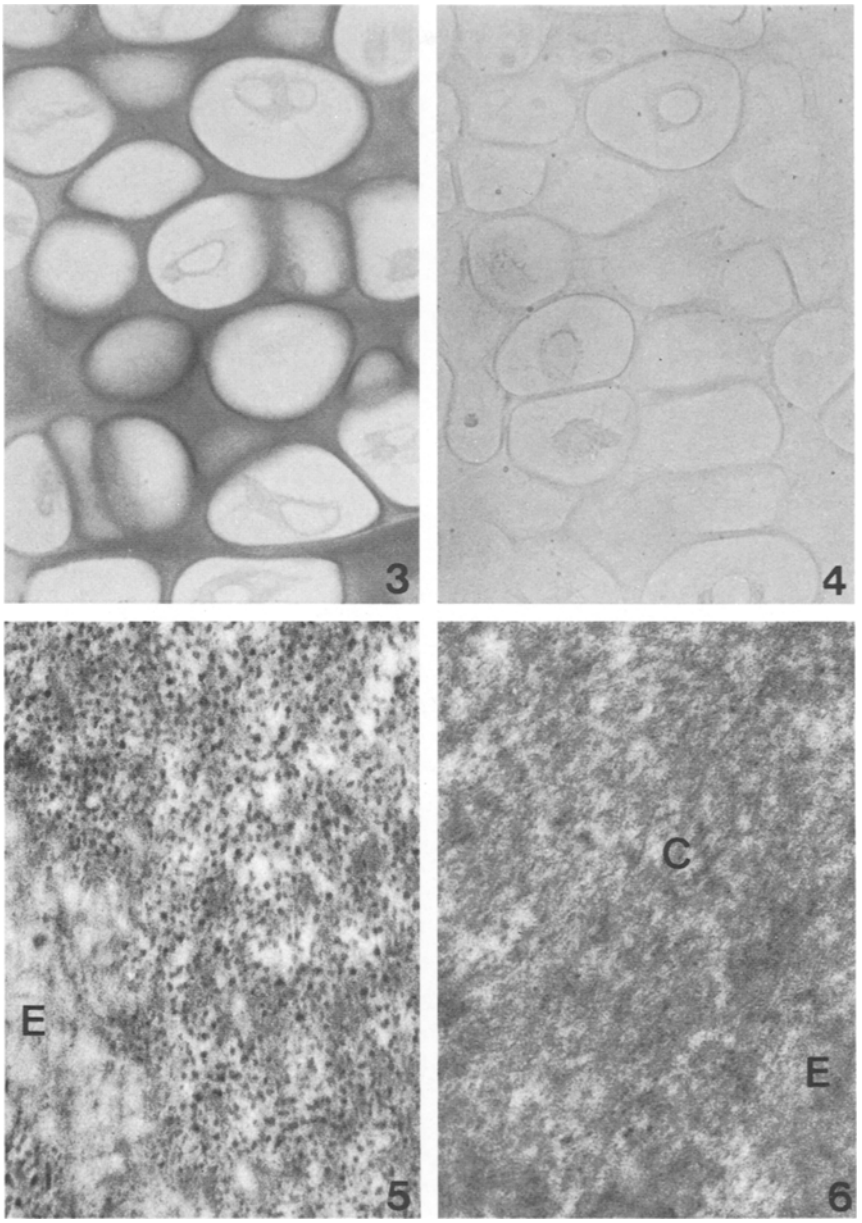


Fig. 3. Normal rabbit ear cartilage. The extracellular matrix shows diffuse basophilia, which is marked immediately around each chondrocyte. Cell are well spaced apart and separated by the matrix. Toluidine blue-staining. ×480

Fig. 4. Rabbit ear cartilage, 12 h after the administration of papain. Loss of basophilia and decrease in amount of the matrix are noted. Chondrocytes are close together. Toluidine blue-staining. ×480

Fig. 5. Normal rabbit ear cartilage, stained with ruthenium red. The extracellular matrix is filled with ruthenium red-positive granules. *E*: elastic fiber. ×40,000

Fig. 6. Rabbit ear cartilage, 24 h after the administration of papain, stained with ruthenium red. Nearly no electron-dense granule is present in the extracellular matrix. *C*: collagen fibril, *E*: elastic fiber. ×40,000

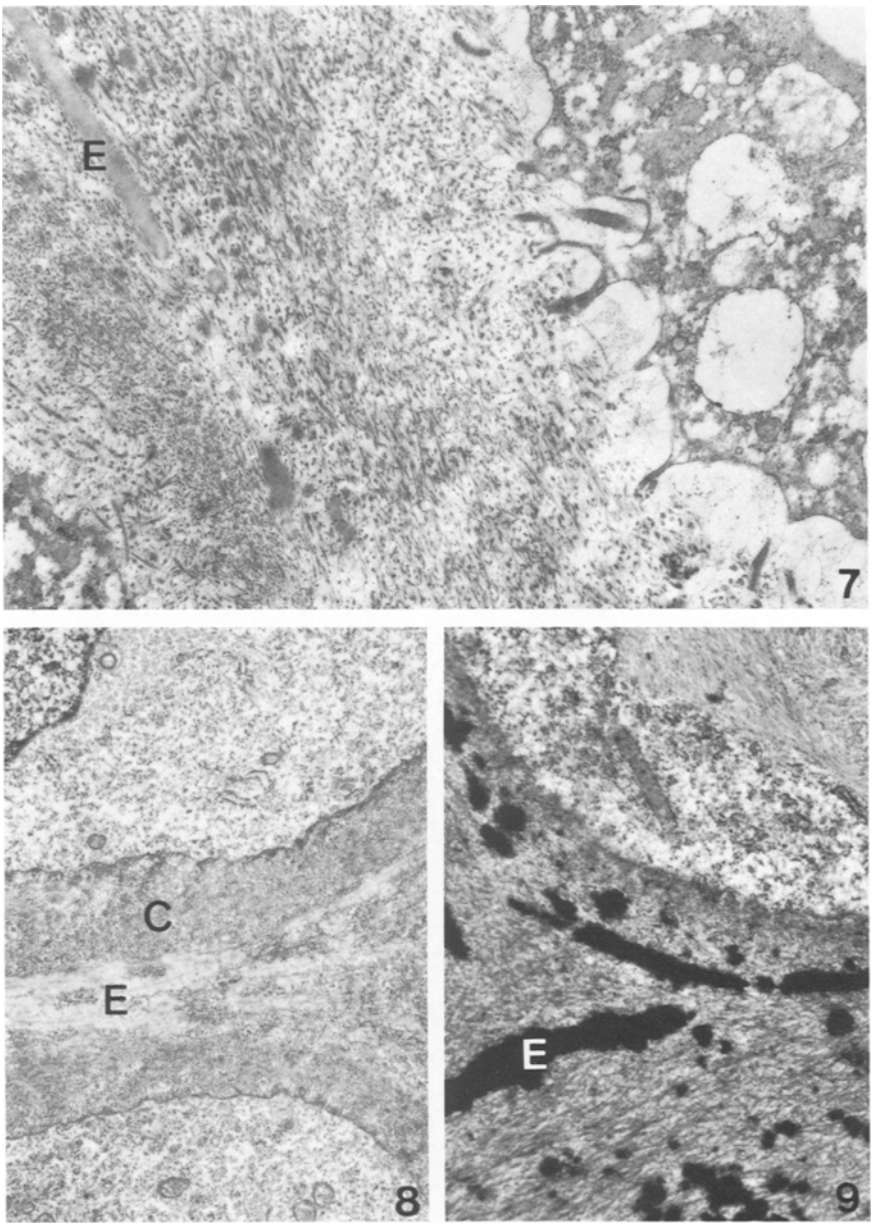
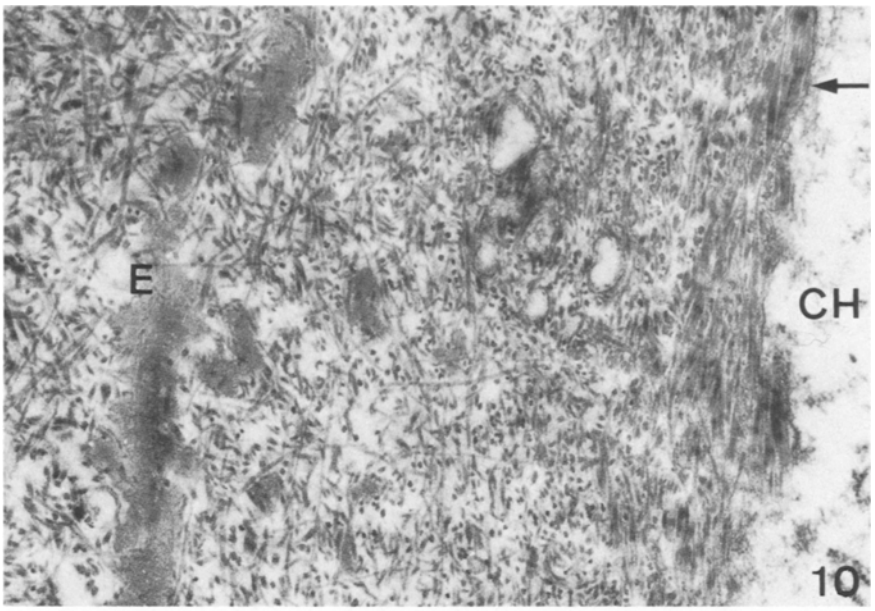


Fig. 7. Rabbit ear cartilage, 4 h after the administration of papain. The cytoplasm of this chondrocyte is markedly vacuolated, and the cell surface is serrated with fine cytoplasmic extensions. The elastic fiber (*E*) shows low electron-density. Collagen fibrils seem to be more or less increased in number. ×10,000

Fig. 8. Rabbit ear cartilage, 8 h after the administration of papain. Chondrocytes are swollen, round and close together. In the oedematous cytoplasm there are a few, small mitochondria and rough-surfaced endoplasmic reticulum, and many free ribosomes. Between the cells there is the narrow intercellular matrix which is filled with many collagen fibrils (*C*), and some electron-lucent elastic fibers (*E*). ×8,800

Fig. 9. Tannic acid-staining section of the identical block, from which Fig. 8 was produced. Elastic fibers which look electron-lucent in Fig. 8 are stained very densely with tannic acid. *E*: elastic fiber. ×10,000



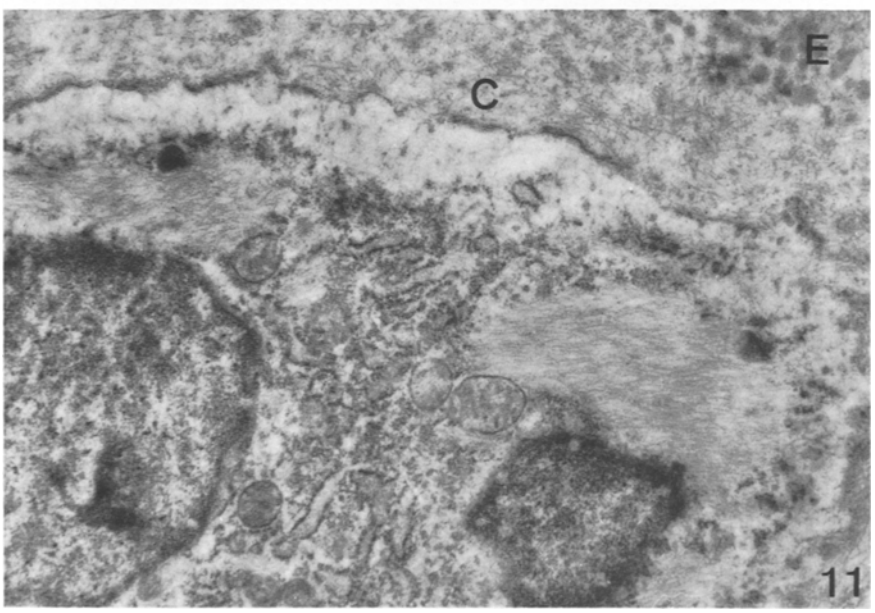


Fig. 10. Rabbit ear cartilage, 12 h after the administration of papain. A chondrocyte (*CH*) is markedly oedematous, and the extracellular matrix is full of entangled collagen fibrils, some of which contiguous to the cell surface assemble into a bundle showing the periodical cross-striation (↑). Elastic fibers (*E*) look moderately electron-dense × 30,000

Fig. 11. Rabbit ear cartilage, 24 h after the administration of papain. A chondrocyte contains well-developed, dilated endoplasmic reticulum and some amounts of tonofilaments. In the extracellular matrix elastic fibers (*E*) begin to be visible. Around the chondrocyte a delicate meshwork of thin collagen fibrils (*C*) is observed. × 14,000

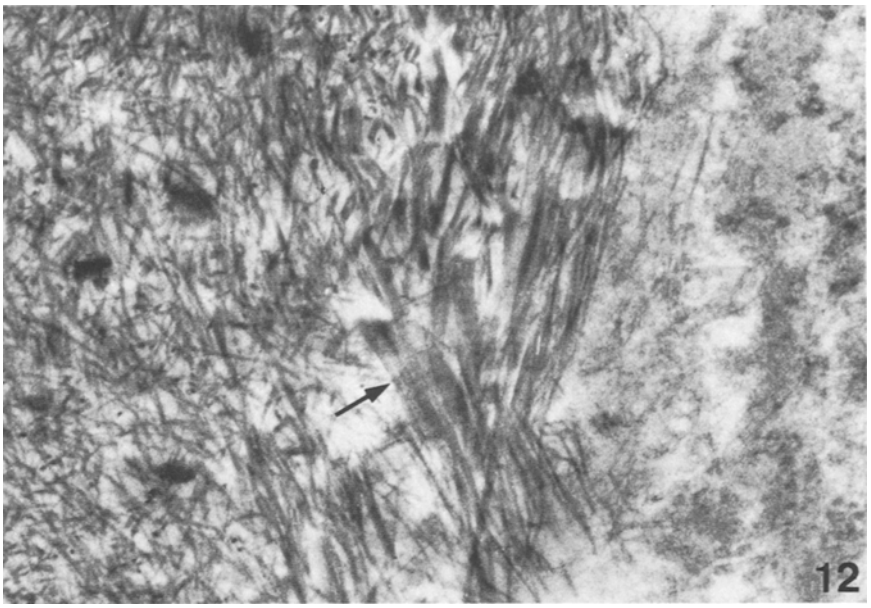


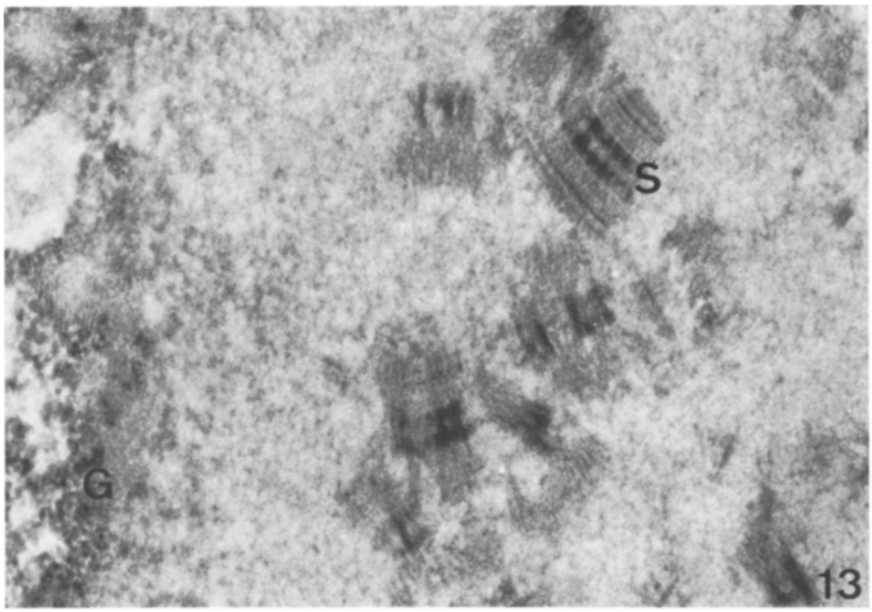
Fig. 12. Rabbit ear cartilage, 96 h after the administration of papain. A part of collagen fibrils lie side by side, showing cross-striation (↑). ×35,000

dilated endoplasmic reticulum and some tonofilaments again (Fig. 11). Thereafter the cell structure returned to the normal form.

b) Extracellular Matrix. In the extracellular matrix of the ear cartilage after the papain treatment, alteration in the electron-density of elastic fibers was noted. About 4 h after the administration of papain, the electron-density of elastic fibers decreased markedly (Fig. 7), and about 8 h later the fibers became nearly electron-lucent (Fig. 8). Such electron-lucent fibers were stained much densely using tannic acid (Fig. 9). From about 12 h and later elastic fibers gradually became dense again (Figs. 10, 11).

In the papain-treated rabbits, the number of collagen fibrils appeared to be increased in the extracellular matrix (Figs. 7–10, 12). In some regions of the ear cartilage, collagen fibrils filled the extracellular space narrowed by swelling and enlargement of chondrocytes lying in close proximity to one another (Figs. 8, 9). A part of the fibrils showed an increase in diameter, up to over 200 Å (Figs. 10, 12). They occasionally appeared to lie side by side and to make bundles with periodical cross-striation (Figs. 10, 12).

The ground substance of the ear cartilage in papain-treated rabbits showed loss of basophilia light-microscopically (Fig. 4). Electron-microscopically, in ear cartilages examined 24 h after the administration of papain, treated with ruthenium red, almost no electron-dense granules were present in the matrix owing to a marked decrease of proteoglycan (Fig. 6). This was in sharp contrast to the findings in the normal ear cartilage stained in the same way (Fig. 5).



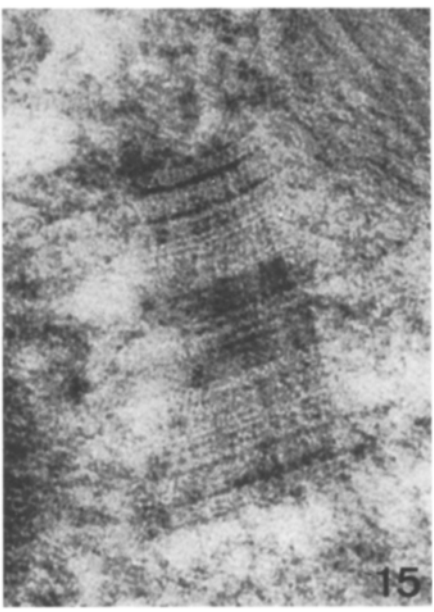


Fig. 13. Rabbit ear cartilage, 72 h after the administration of papain. Near a chondrocyte containing many glycogen granules (*G*), there are some ribbon-like collagen segments with an atypical FLS-type of cross-striation (*S*). × 50,000

Fig. 14. Rabbit ear cartilage 24 h after the administration of papain. In the extracellular space, there are collagen segments with an incomplete pattern of atypical cross-striation. × 35,000

Fig. 15. Higher magnification of the cross-striated segment in the identical block, from which Fig. 13 was obtained. It has the symmetrical cross-striation, a pair of electron-dense broad bands at the center and two darker striae near to both ends. × 130,000

c) Cross-Striated Collagen Segments of an Atypical FLS-Type. In one of the experimental animals treated with papain, ribbon-like, collagen fibrous segments with an atypical cross-striation were found near the chondrocytes in the ear cartilage during the period in which the collapsed ear was recovering (Figs. 13–15); we observed these collagen segments in specimens taken successively from the same animal at 24, 48, 72 and 96 h after papain administration. The collagen segments seen in the ear cartilage at 24 h after the papain treatment had an immature form of cross-striation (Fig. 14). The segments appearing after 72 h showed a distinct pattern of cross-stripes (Figs. 13, 15). They were characterized by symmetrical cross-striation and had a length of about 3,500 Å, along the axis perpendicular to these striations. At the center of the segment there were a pair of electron-dense broad bands, about 300 Å thick each, with a less-dense band of the same thickness in between. Near to both ends were found two darker and thicker striae; these dark striae were located about 300 Å apart from each other. The distance between the outer dark striae on the one side and that on the other side, was about 3,000 Å. At higher magnification more than 40 stained cross-stripes of about 40–60 Å thick were distributed on the segment and they were also discernible within the central broad bands. After 96 h the collagen segments in the ear cartilage were decreased and looked indistinct in the cross-banded pattern.

Discussion

Sheldon and Robinson (1960) first observed the cartilage of collapsed ears of papain-treated rabbits electron-microscopically. They described cytoplasmic vacuolization and a ruffly appearance of the cell membrane in the chondrocytes of the rabbit ear during the early period after the administration of papain. In this experiment, however, many cartilage cells in the rabbit ear became oedematous and swollen during the same period, and the cell surface came to be rather smooth. In some cartilage cells, the cytoplasm was remarkably vacuolated and the cell membrane was much serrated, as reported by Sheldon and Robinson. Since the morphological appearance and the functional situation of each chondrocyte are not uniform even in the normal state of the cartilage, the papain treatment may further intensify the ultrastructural differences among these cells.

Tsaltas (1958) suggested that collapse of the rabbit ears by papain was due to a marked decrease of proteoglycan in the ear cartilage. Shepard and Mitchell (1977) showed that the rabbit articular cartilage previously treated with papain in vitro was not stained by ruthenium red. Absence of ruthenium red-positive granules in the cartilage of the collapsed ear (Fig. 6) reflects loss of proteoglycan by papain digestion. In the ears of young rabbits given crude papain, the electron-density of elastic fibers in the ear cartilage was decreased markedly initially and afterwards recovered. About 8 h after the papain administration the elastic fibers became nearly electron-lucent (Fig. 8). Sheldon and Robinson (1960) mentioned a virtual absence of the elastic fibers from the

matrix between the chondrocytes of the rabbit ear cartilage during the same period after the papain treatment. Our electron micrographs, however, did not show an actual disappearance of the elastic fibers in the ear cartilage during that period, but only loss of their stainability by the regular fixative and staining techniques (Fig. 8). The electron-lucent fibers were stained much more densely by tannic acid (Fig. 9). A decrease in the electron-density of the elastic fibers seemed to run parallel with loss of proteoglycan from the cartilage matrix.

The assembly and growth of collagen fibrils in vitro and in vivo are altered by the presence of proteoglycan or acidic glycosaminoglycan. The initiation of collagen nucleation is enhanced by the presence of proteoglycan or acidic glycosaminoglycan, but subsequent growth of the fibrils is inhibited by their presence (Seegmiller et al. 1971). Collagen fibrils in the normal ear cartilage are very thin, less than 200 Å in diameter, without periodical cross-banding. In the cartilage matrix, polymerization of collagen molecules and growth of collagen fibrils are limited by large amounts of proteoglycan or acidic glycosaminoglycan. In this study, removal of proteoglycan from the cartilage matrix by papain digestion, probably brought about the assembly as well as the unmasking of collagen fibrils. Therefore, in the extracellular space, collagen fibrils appeared to be increased in number, and some of their diameters became greater (more than 200 Å). Some of thicker fibrils assembled into bundles, in places, accompanied by the periodical cross-striation.

The collagen fibrous segments with an atypical cross-striation in Figs. 13–15 are possibly a kind of FLS (fibrous long spacing) fiber; collagen molecules may be arranged in an antiparallel way and staggered alternately on both sides by some distance. The collagen segments are very similar, in the cross-banded pattern, to those which Merker et al. (1978) obtained by addition of SP 54 (pentosanpolysulfoester, molecular weight 2,000 daltons, Benechemie) or Arteparon (mucopolysaccharidepolysulfoester, molecular weight 12,000, Luitpold-Werke) to the tissue cultures of limb buds from 11-day-old mouse embryos. They reported that the appearance of these atypical collagen segments after application of SP 54 or Arteparon was only related to type II collagen which was abnormally aggregated by the highly sulfated substance. Yet no one has ever seen this type of structure in tissues in situ. In the production of this type of segment in our experiment some kind of endogenous sulfated carbohydrate may have acted to produce the atypical arrangement of type II procollagen or collagen newly produced by the recovering chondrocytes in the ear cartilage. It is not known why the segmental structure did not appear in every experimental animal in this study. The specific conditions necessary for production of the structure in the cartilage matrix may exist in a particular rabbit with some abnormality of the connective tissue. Merker et al. reported that when highly sulfated glycosaminoglycans were removed from the culture, these structures disappeared within 3 days. In our case the structures, which showed a very distinct pattern of cross-banding at 72 h after the papain treatment, were decreased and looked indistinct in the cross-striation after 96 h. Around this time the highly sulfated substance concerned probably decreased in amount in the cartilage matrix of the ear.

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