Virchows Arch (2000) 436:74–81

CASE REPORT

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A morphological study of bone and articular cartilage in ochronosis

Received: 17 May 1999 / Accepted: 24 June 1999

Abstract An ochronotic femoral head has been studied morphologically under the light and the electron microscope. Its articular cartilage showed the alterations already reported in the literature, mainly consisting of erosions of the surface, pigment accumulation in chondrocytes and intercellular matrix, chondrocyte degeneration, the formation of pigmented, calcified and uncalcified microshards, and the presence of granulation tissue with macrophagic cells. The changes in bone were less severe than those in cartilage. Pigment was present in the calcified matrix. This did not seem to disturb the organization of the bone tissue, although it was diffusely osteoporotic, perhaps because of limb disuse. The preservation of calcified matrix might depend on the fact that its collagen fibrils are encrusted by mineral substance, which avoids the dangerous effects that the deposition of ochronotic pigment induces in the fibrils of soft connective tissues. On the other hand, the newly formed osteoid matrix remains uncalcified for too short a time to be modified by the pigment. Diffuse or granular pigmentation was found in a few osteocytes, while several of them were condensed or reduced to cellular fragments. Bone resorption often occurred near these osteocytes. However, this did not seem to alter the degree of bone remodelling, possibly because of the relatively low numbers of degenerated or dead osteocytes. Pigment was also contained in the cytoplasmic vacuoles of otherwise active osteoclasts, whereas it was not found in osteoblasts. On the whole, ochronosis in bone seems to induce the same changes as in other connective tissues. However, their severity appears to be limited by calcification, which prevents modifications in collagen fibrils, and by bone remodelling, which to some extent eliminates the oldest, pigment-richest parts of the tissue.

Key words Ochronosis · Bone · Cartilage · Arthropathy · Alkaptonuria

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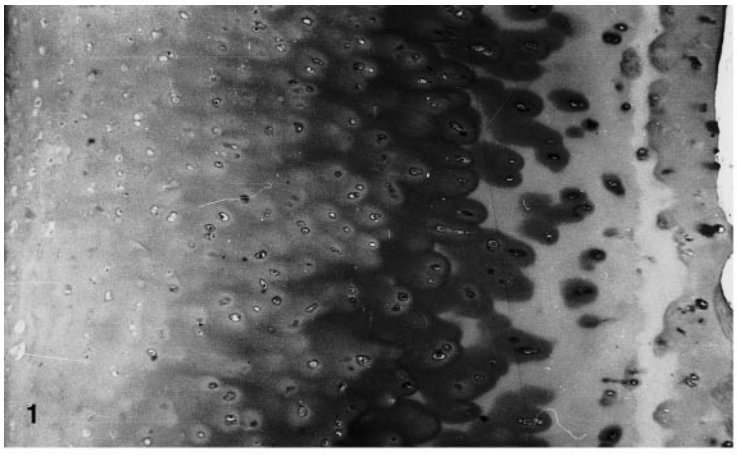
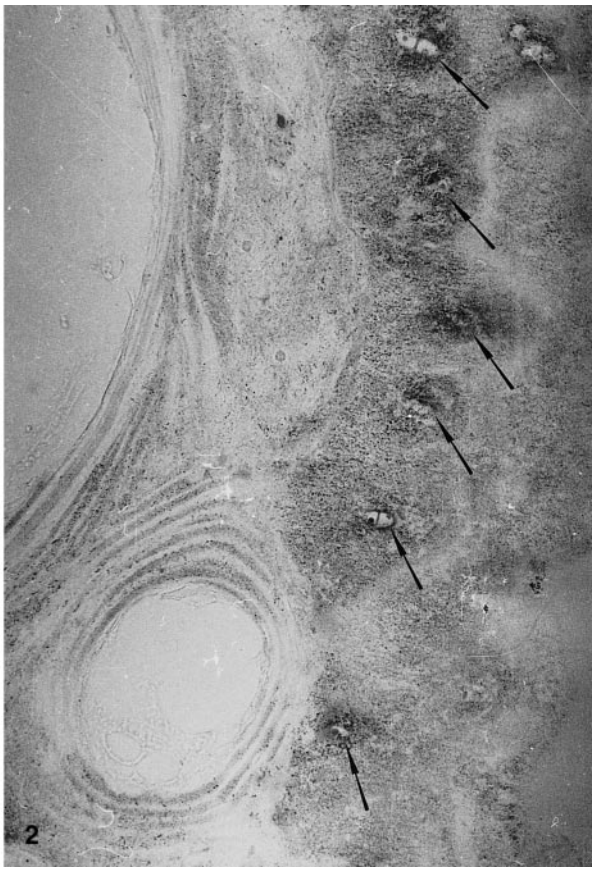
Introduction

Ochronosis is the major tissue change produced by alkaptonuria, an autosomal recessive inborn error of the metabolism which is due to excessively high concentrations of homogentisic acid in the body as a result of homogentisic acid oxidase deficiency [1]. The acid is excreted in the urine, which turns dark on exposure to air. Moreover, it is deposited as a brown to yellowish, ochronotic pigment in all connective tissues, but especially in cartilage; these acquire a brown colour. In spite of the relative rarity of the disease, its inheritance, clinical manifestations, pathology, basic defects, and pathogenesis are well known [1, 6]. All the collagenous components of the tissue may be altered, but cartilage is the most severely affected: the deposition of ochronotic pigment in articular cartilage leads to its brittleness, fissuring and fragmentation, with the development of severe ochronotic arthropathy. Cartilage changes due to deposition in the cells and matrix of ochronotic pigment have been noted in a number of case reports [5–7, 12, 13, 16–18]. On the other hand, changes in bones have been neglected, perhaps because they induce less disabling symptoms, or because specimens of ochronotic tissue are frequently obtained during arthroscopy, which only provides samples of synovial and articular cartilage, or because of the well-known difficulties encountered in morphologic studies of calcified matrices. For these or other reasons, only short outlines of ochronotic skeletal alterations are available in the literature [6]. We have had the opportunity of studying a femoral head which had been surgically resected and substituted with a prosthesis because of severe ochronotic arthropathy. This paper re-





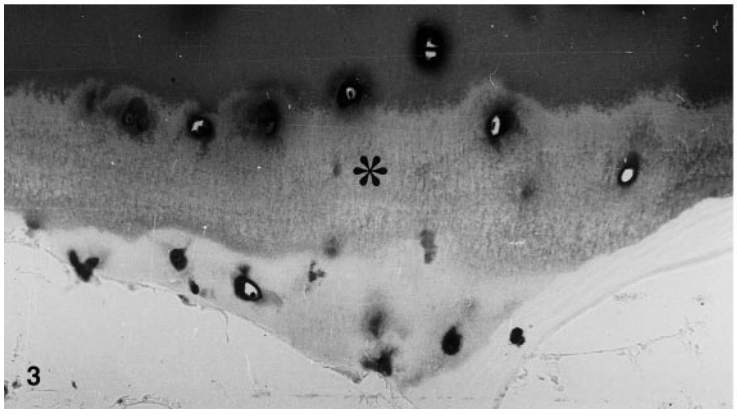


Fig. 1 A well-preserved zone of articular cartilage of the femoral head (articular surface on *left*); approximately the outer third of the matrix is lightly basophilic, the central third is deeply basophilic, the deepest third is eosinophilic. Haematoxylin–eosin, ×65

Fig. 2 Metaphyseal bone (*left*) and the deepest layer of articular cartilage (*right*): the cartilage matrix, the chondrocytes (*arrows*), and some of the osseous lamellae all contain very fine, brown to yellowish granules of ochronotic pigment. Unstained, ×190

Fig. 3 Articular cartilage (*above*) and metaphyseal bone (*below*): the chondrocytes and some of the osteocytes are deeply stained; the *asterisk* shows the calcified layer of the cartilage. Cresyl violet, ×190

ports the histological and ultrastructural changes found not only in the articular cartilage of this femoral head, but above all in its spongy bone.

Clinical history

A 46-year-old woman was admitted to the Rheumatology Unit of the Department of Medical Therapy in the "La Sapienza" University of Rome with a history of severe, disabling left coxalgia dating back to 1996. The patient did not report any previous serious diseases or metabolic disorders. Her family history was negative for genetic or heritable diseases. The patient was 162 cm tall and her body weight was 62 kg.

She had brown discoloration of the sclerae and auricles. Her spine was moderately rigid, while abduction of her left hip was almost impossible. Her urine turned dark after the addition of an alkali, and homogentisic acid was detected in it by gas chromatography at a concentration of 1586 µmol/mmol creatine (range 1000–5000 µmol/mmol creatine). The radiographic examination showed focal and diffuse osteoporosis of the pubis and the femoral heads, especially the left one, sclerosis of the left acetabulum with shrinkage of the articular space, and "wafer-like" calcification of intervertebral discs of the lumbar tract of the spine, with moderate osteoporosis of the vertebral bodies. No abnormalities were detected in the usual metabolic parameters.

The patient was diagnosed as having ochronotic arthopathies and was treated with nonsteroidal anti-inflammatory drugs, which relieved her arthralgia almost completely. She was asymptomatic for about 1 year, after which she began suffering severe pain in both hips. Eventually, the pain in her right hip became worse, leading to almost complete functional disability. The radiographic examination showed osteonecrosis and moderate arthrosis of the left femoral head, reduction of the hip articular space on right, and osteoporosis on both sides. The MRI showed a necrotic area in the subchondral bone of the left femoral head, diffuse neuro-algodystrophic changes, with the presence of subchondral microcysts in both femoral heads, reduction of the hip articular space, and a rise in the tension of the right articular capsule. The patient underwent a surgical operation for the prosthetic replacement of the left femoral head.

Materials and methods

The surgically resected specimen consisted of the head and a small portion of the neck of the left femur. The articular cartilage was brown to yellowish, and its surface was irregular because of deep erosions. The specimen was divided into two parts along a frontal plane passing through the ligament insertion. The examination of the cut surface showed an approximately triangular, rather amorphous, pale zone delimited by a brown to reddish border, with the base at the surface of the articular cartilage and the apex almost reaching the centre of the head. This zone seemed to

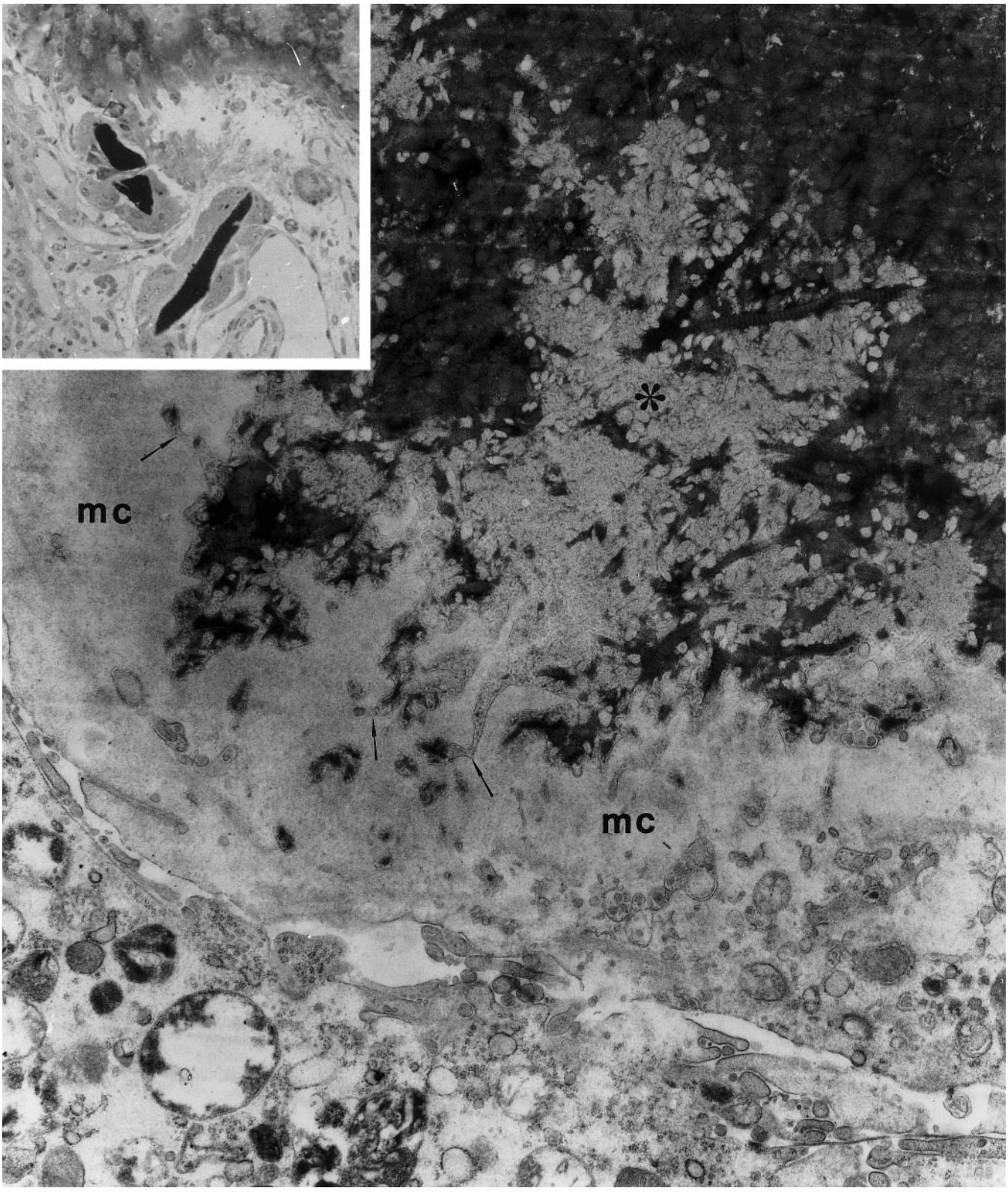
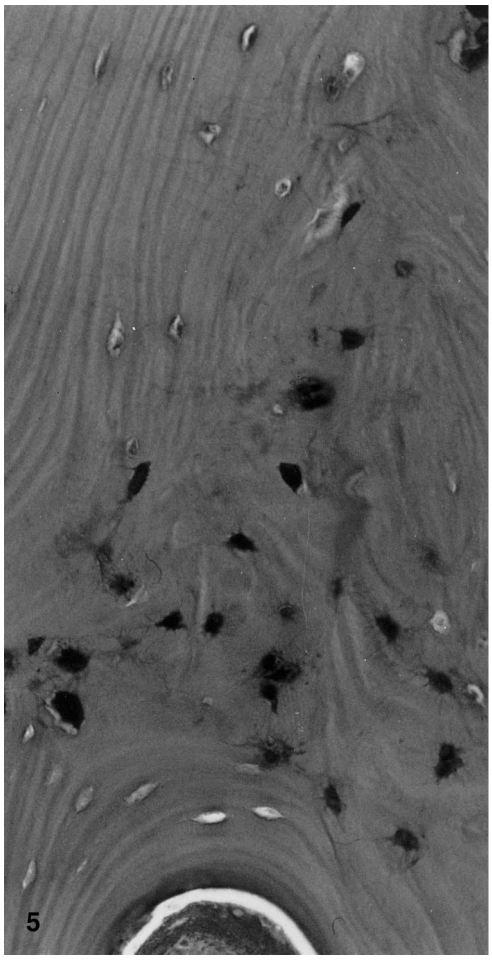
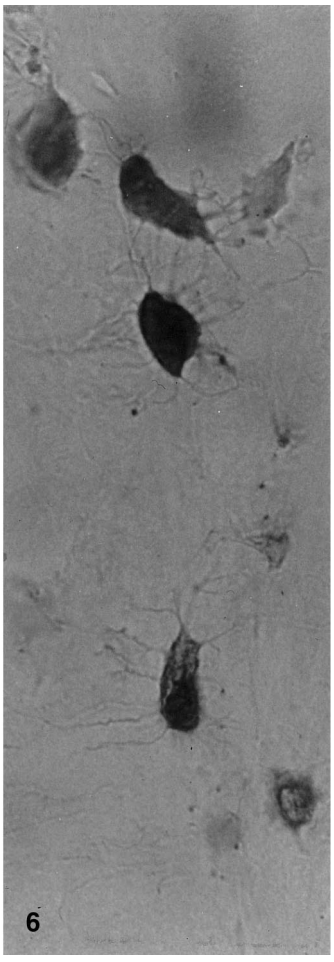


Fig. 4 Two cartilaginous microshards are shown in the *inset*: note their deeply stained matrix, and the surrounding macrophagic cells; newly formed bone above. Haematoxylin–eosin, ×190 Ultrastructure of part of the cartilage microshard and surrounding macrophagic cells shown in the *inset*: the microshard has a highly electron-dense matrix where this has not been modified by the degrading activity of the macrophage (*upper right*), whereas dissociated collagen fibrils, empty vesicles, and very fine granules are visible in the degraded zone (*asterisk*). The cytoplasm of the macrophagic cell (*mc*) forms a coarse brush-border whose canaliculi (some shown by *arrows*) contain dissociated components of the cartilage matrix; electron-dense material, presumably ochronotic pigment, is recognizable in the cytoplasm of another macrophagic cell, partly visible below. Uranyl acetate and lead citrate, ×20,000





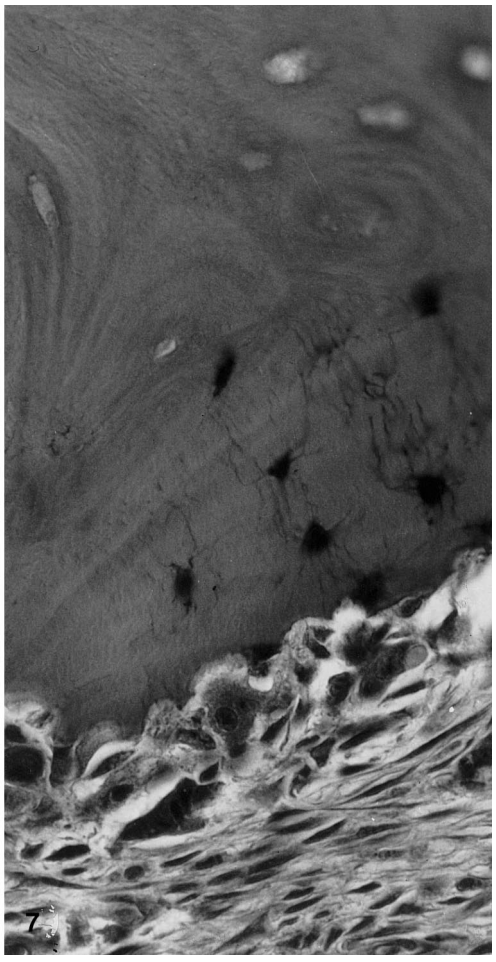


Fig. 5 Lamellar and woven bone in a metaphyseal trabecula: the osteocytes of woven bone are deeply stained. Haematoxylin–eosin, ×300

Fig. 6 Osteocytes containing variable amount of ochronotic pigment (brown to yellowish under the microscope). Unstained, ×750

Fig. 7 Detail of a metaphyseal trabecula: deeply stained osteocytes are present in a zone of the matrix that is undergoing osteoclastic resorption. Haematoxylin–eosin, ×375

correspond to the infarcted area revealed by radiographic examination.

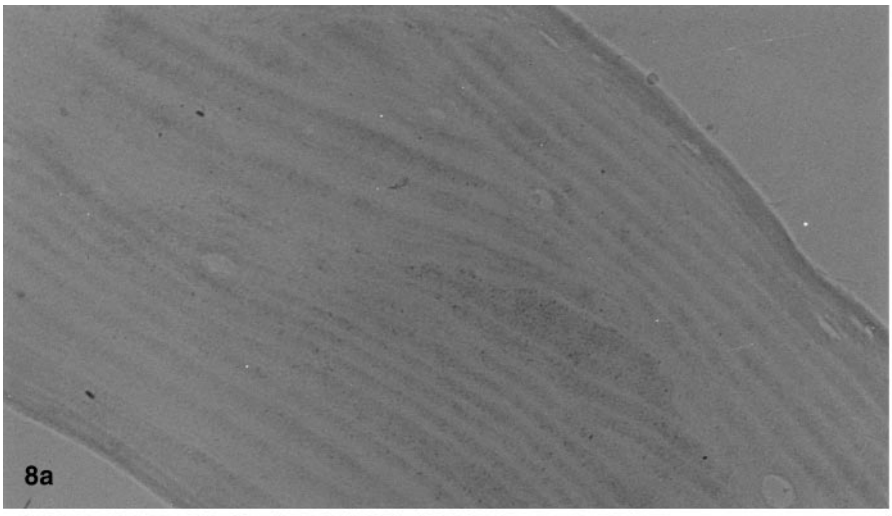
Specimens of the femoral head were cut outside the triangular zone in such a way as to obtain articular cartilage and spongy bone in the same specimen. They were fixed for 24 h in 4% formaldehyde buffered at pH 7.2 in 0.1 M phosphate buffer, and were then decalcified in a repeatedly renewed solution of equal parts of 5% nitric acid and 5% ammonium oxalate in distilled water. After further fixation in buffered formaldehyde, they were dehydrated and embedded in paraffin. Sections were stained with haematoxylin and eosin for routine examination, periodic acid-Schiff (PAS) for glycoproteins, or 1% alcian blue, pH 2.5, for acid proteoglycans; they were also stained with cresyl violet, which gives the ochronotic pigment a blue-black colour [19]. Sections were also examined unstained by both light and polarizing microscopy.

Additional parts of the femoral head were cut into small fragments in such a way that some of them corresponded to spongy bone and some to spongy bone and articular cartilage. These specimens were fixed for 2 h in 2% glutaraldehyde buffered at pH 7.2 with 0.1 M cacodylate buffer . Some of them were decalcified for 12 h with 4% EDTA, and some were left undecalcified. After washing in distilled water, they were post-fixed for 2 h with 1% osmium tetroxide buffered as above, washed in distilled water, dehydrated in increasing concentrations of ethanol, passed through propylene oxide, and embedded in Araldite. Semithin (about 1 µm thick) sections were stained with azure II–methylene blue for routine examination, or were treated with von Kossa's method for calcium phosphate. Ultrathin sections (about 75 nm thick) were examined under the electron microscope either untreated or after treatment with uranyl acetate and lead citrate.

Results

Under the light microscope, the articular cartilage was seen to have undergone various degrees of alteration. It appeared almost unchanged in some places, where only slight irregularities of its surface showed its abnormal state; it had completely disappeared in other places, so that the articular surface showed deep erosions whose base consisted of irregular osseous trabeculae, granulation tissue, and fragments (or "microshards") of articular cartilage; intermediate degrees of alterations were visible in other places.

In the least altered zones, the articular cartilage showed a homogeneous matrix with a fall in numbers of chondrocytes. The staining properties of the matrix were variable (Fig. 1): roughly dividing the cartilage into three layers, the shallowest was lightly basophilic (i.e., stained



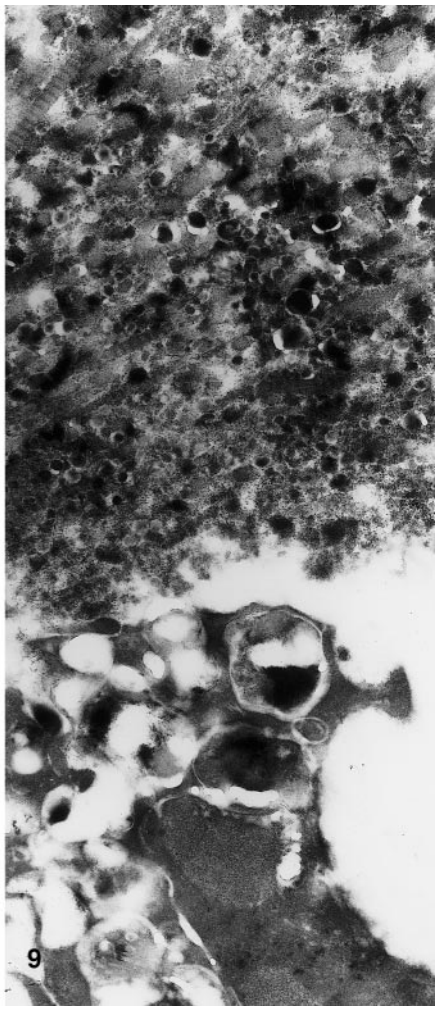


Fig. 8. The same unstained section examined by **a** light and **b** polarizing microscope: many of the lamellae have a granular appearance because they contain ochronotic pigment (they are brown to yellowish under the light microscope); the same lamellae are isotropic by polarizing microscope. ×190

Fig. 9 Electron microscope picture of articular cartilage, showing part of a degenerate chondrocyte (*below*) and of its territorial matrix (*above*): a number of roundish structures of variable electron density are scattered through the collagen fibrils of the dense matrix. Uranyl acetate and lead citrate, ×17,000

by haematoxylin) and alcianophilic, and was PAS positive; the central layer was deeply stained by haematoxylin and PAS, whereas alcian blue stained only the matrix around the chondrocytes (territorial matrix); the deepest layer was stained by eosin, but not by alcian blue, though it was stained by the PAS method, but only in the territorial matrix. The brown to yellowish colour visible in the cartilage during the macroscopic examination was still visible under the light microscope in unstained sections (Fig. 2); pigmentation was especially abundant in the deepest, von Kossa-positive (i.e., calcified) zone of the cartilage, where chondrocytes showed minute, intralacunar granules or appeared so homogeneously condensed that their morphology was no longer recognizable (Fig. 2). The granules were stained by cresyl violet (Fig. 3), whereas they were no longer recognizable after PAS or alcian blue staining.

Distorted osseous trabeculae were present where the articular cartilage was eroded. They were surrounded by granulation tissue consisting of collagenous fibres, mononucleated cells, macrophagic cells and capillary vessels, and showed several resorption lacunae with or without osteoclasts. Cartilaginous microshards of variable dimensions were irregularly scattered through the granulation tissue (Fig. 4, inset). They were often surrounded by macrophagic cells. Their matrix was deeply stained by cresyl violet, but not by alcian blue, and showed varying degrees of PAS positivity. They appeared deeply pigmented (brown to yellowish) in unstained sections.

The spongy bone consisted of thin, irregular osseous trabeculae, which delimited bone marrow spaces that were wider than normal and contained fat cells or bundles of thin collagen fibrils. Most of the trabeculae had smooth margins lined with so-called lining cells. Bone remodelling could, however, be observed in several areas, especially those adjacent to cartilage erosions: in this case, some trabeculae showed focal osteoclastic resorption and others osteoblastic reconstruction. The two processes were sometimes present in the same trabecula.

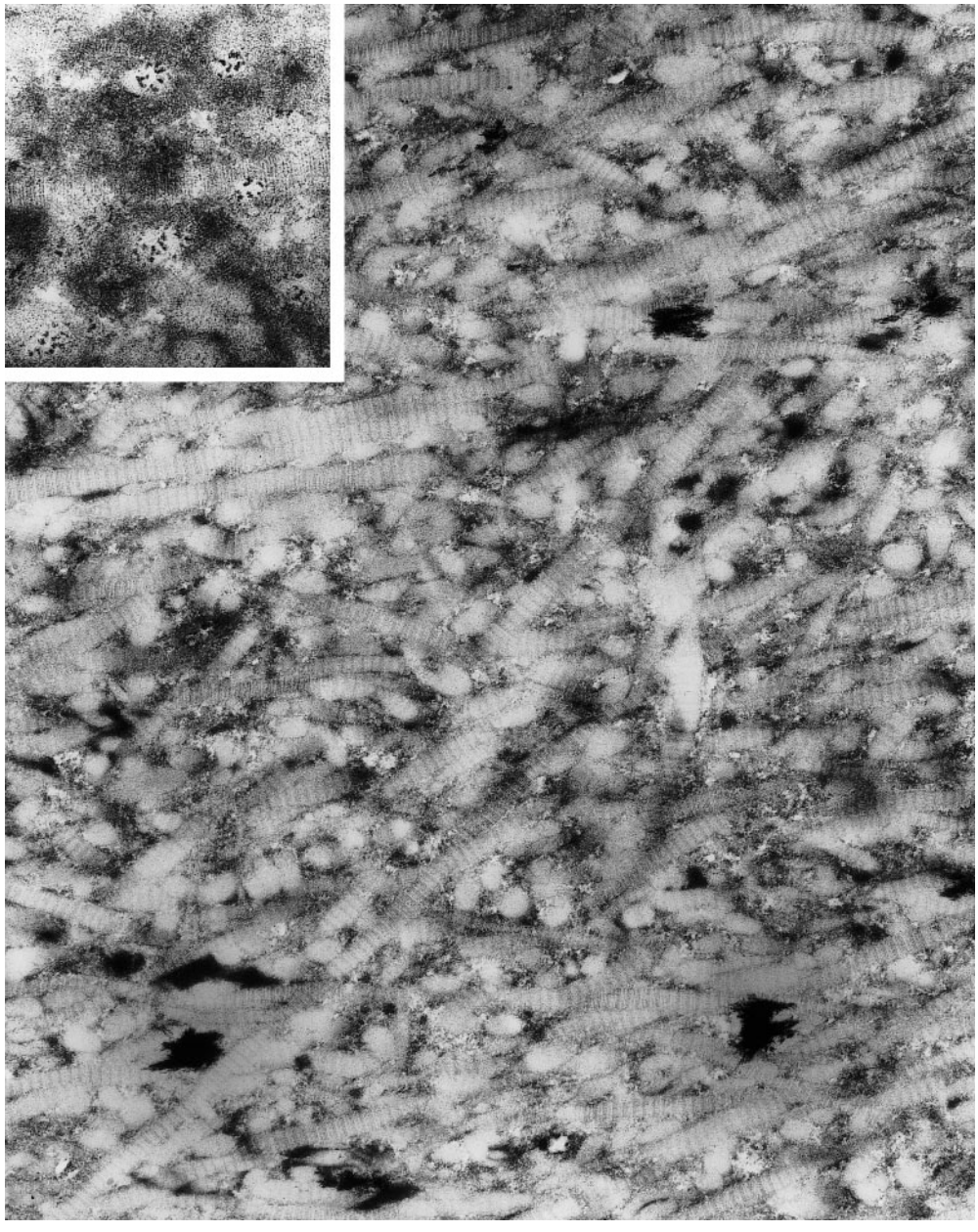


Fig. 10 Ultrastructure of the deepest layer of the articular cartilage: thick, irregularly interwoven collagen fibrils are visible; the electron-dense deposits, like the very small interfibrillary granules shown in the *inset*, presumably correspond to ochronotic pigment. Uranyl acetate and lead citrate, ×27,000; *inset* ×30,000

Most osteocytes showed no morphological changes. When examined in unstained sections, however, some of them were diffusely brown to yellowish (Fig. 6), or showed black granules in their cytoplasm and at the border of their lacuna. These osteocytes appeared deeply stained by haematoxylin (Figs. 5, 7) or cresyl violet (Fig. 3), and showed PAS positivity greater than that of the unaltered osteocytes, whereas they were not stained by alcian blue. Several osteocytes were condensed or reduced to cellular fragments. Osteoclastic bone resorption often occurred near pigmented or degenerate osteocytes (Fig. 7).

A weak brown to yellowish colour, or very fine black granules, were recognizable over some of the lamellae in unstained sections of lamellar bone (Figs. 2, 8a). When viewed with the polarizing microscope, the pigmented lamellae were found to be mostly the isotropic ones (Figs. 8a, b). They were lightly stained by cresyl violet.

Both the osteoclasts, and the giant macrophagic cells found in bone marrow spaces and in the granulation tissue, especially around the cartilage microshards, contained brown to yellowish granules in their cytoplasm.

Undecalcified semithin sections showed the same type of changes as those just described. Von Kossa's method showed that some of the microshards were uncalcified, whereas others were uniformly calcified.

In the electron microscope (Figs. 9, 10), the cartilage matrix was characterized by thick collagen fibrils and a granular or amorphous substance which filled up all interfibrillar spaces. In some areas these were occupied by plenty of roundish, electron-dense bodies (Fig. 9). Moreover, small aggregates of highly electron-dense granules

were located between the fibrils and on their surface. Chondrocytes very often appeared degenerated (Fig. 9): in many cases they had changed into irregular aggregates of granular and amorphous material, corresponding to the ochronotic pigment seen under the light microscope, in which no cell remnants could be found; or their cytoplasm was preserved to some extent but contained roundish aggregates of the same material. These aggregates varied in shape and size and appeared to be surrounded by a single membrane.

The ultrastructural details of the microshards were hard to make out because of their high electron density, compactness and homogeneity. They were best studied where a microshard was undergoing resorption by macrophagic, osteoclast-like cells, so that its matrix appeared lightly dissociated (Fig. 4). The giant, multinucleated cells that surrounded the microshards were in close contact with its matrix through inconspicuous ruffled borders; in these zones, the matrix appeared partly disaggregated, so that its components were recognizable. These consisted of electron-dense collagen fibrils, fragments of collagen fibrils, highly electron-dense amorphous or granular substance, and lightly electron-dense, roundish structures which gave the matrix a vacuolated appearance. These last two components were also found in the canals of the ruffled border and within cytoplasmic vacuoles. Ochronotic pigment was contained in other cytoplasmic vacuoles.

The many macrophagic cells present in the granulation tissue and bone marrow spaces contained varying amounts of ochronotic deposits. These were located in roundish or irregular vacuoles lined by a single membrane, and appeared as aggregates of electron-dense, amorphous or granular material.

The osteoblasts and osteoclasts present in the areas of bone remodelling were no different from those usually found in normal bone [14]. Most of the osteocytes also appeared to be normally structured, resembling those found in woven and lamellar bone [3]. Some osteocytes, however, appeared to be condensed into amorphous aggregates in which nuclear fragments and cytoplasmic organelles were hardly recognizable. Some of the lacunae contained roundish aggregates of granular material.

In fully calcified areas, the bone matrix was covered by mainly unoriented inorganic crystallites, which completely masked its organic components. After decalcification, collagen fibrils with a period of about 700 nm were recognizable. Roundish or irregular, amorphous or granular, strongly electron-dense patches appeared between them. These patches, which had resisted decalcification, can be interpreted as interfibrillar aggregates of ochronotic pigment.

The matrix of the deepest layer of the articular cartilage showed variable degrees of calcification. Where this was complete, the organic components were completely masked by crystallites, and ochronotic pigment could not be differentiated from them. Where calcification was incomplete, small, roundish or irregular, electron-dense patches were present. Some of these patches were recognizable as calcification nodules because of the presence of crystallites; others, whether granular or amorphous, resembled those found in bone matrix after decalcification, and corresponded to aggregates of ochronotic pigment (Fig. 10). Crystallites, granules, and amorphous material were also scattered at random through the matrix.

Discussion

The ochronotic changes found in articular cartilage did not differ from those reported in the literature [12, 16–18]. The cartilage surface was irregular and showed erosions. Its chondrocytes had degenerated to varying degrees; some of them appeared to have disintegrated or to have been replaced by amorphous or granular substance. The matrix contained ochronotic pigment and a number of roundish electron-dense bodies; these resembled matrix vesicles [2] and/or lipid bodies [8]. The haematoxylin, PAS, or alcian blue staining of the matrix showed that the articular cartilage could be roughly divided into three zones characterized by different distributions of glycoproteins and acid proteoglycans. These findings, however, were similar to those described in ageing hyaline cartilage [4] and did not appear to depend on ochronosis. Even so, the degenerative appearance of the chondrocytes was closely linked to the accumulation of ochronotic pigment in their cytoplasm, as shown by electron microscopy and by their being stained with cresyl violet. Ochronotic pigment was certainly present in cartilage matrix, as previously reported [6]. However, not all granular material on and between collagen fibrils corresponded to ochronotic pigment. Although their presence varies quantitatively and qualitatively with the zone of articular cartilage, proteoglycan granules are demonstrable on and between collagen fibrils by electron microscopy [9], and some of them could be confused with ochronotic pigment. Moreover, in calcified cartilage matrix, differentiation between calcification nodules and pigment aggregates may be difficult or impossible.

The presence of cartilage microshards has been described in all the cases of ochronotic arthropathy studied so far [6, 11]. They represent fragments of degenerate cartilage that has probably been traumatically detached from the articular surface or been left behind by incomplete erosion. They, as well as the deepest zone of the cartilage, may be calcified, but most of them are uncalcified. Their ultrastructure is different from that of articular cartilage, because of the presence of an amorphous, highly electron-dense substance that masks the other components of the matrix. Where the microshard is resorbed by macrophagic cells its matrix appears dissociated into three components: collagen fibrils, similar to those found in articular cartilage; amorphous and granular aggregates, probably corresponding to ochronotic pigment; and unidentified roundish structures, which might correspond to the roundish structures found so often in the matrix.

Bone tissue was not greatly altered, although it was diffusely osteoporotic. Osteoporosis was probably not due to ochronosis itself but, more precisely, to the impairment of limb function consequent on the ochronotic arthropathy of the hip. However, ochronotic pigment was found in the bone matrix. It is reported that the accumulation of ochronotic pigment in connective matrices induces rigidity in their structures, which become brittle and may fracture [1]. This is certainly true of cartilage, but probably not of bone, whose matrix is rigid simply because it is highly calcified. Thus, the presence of the pigment might have no appreciable effects on bone matrix, whose mechanical properties and resistance to load or traumas remain the same. It is interesting that the pigment appears to be preferentially deposited in isotropic (not birefringent) lamellae. This might be due to a preferential localization of the pigment in lamellae with looser texture and greater amount of interfibrillar material [15]. As the newly formed osteoid matrix is uncalcified, it could be exposed to the dangerous effects of the ochronotic pigment; however, it remains free of mineral for too short a period to be altered by the slow process of pigment deposition.

Ochronotic pigment was found not only in bone matrix, but also in osteocytes. These showed either diffuse brown to yellowish pigmentation of the cytoplasm, or the intralacunar accumulation of dark granules of pigment. In the second case, some of the osteocytes had been disrupted, and had probably died. It is difficult to determine the effect these changes may have on the functionality of the tissue and its remodelling. Because they affect limited numbers of osteocytes, they probably have no dangerous effects on the tissue as a whole. After all, some osteocytes degenerate and die even under normal conditions [10, 20]. On the other hand, osteocytes are essential for preserving tissue integrity, so that pigment accumulation even in a small proportion of them might contribute to an impairment of their role in maintaining the physiological state of the tissue, and might promote bone resorption.

Accumulation of pigment granules was also found in macrophagic cells present in the granulation tissue and in bone marrow spaces. Their cytoplasm was full of phagocytosed pigment granules. These resembled those previously described in synovial cells and in macrophages [7, 12]. A small number of granules were also found in osteoclasts. They could have been gathered from the bone matrix during its resorption.

In conclusion, the deposition of ochronotic pigment occurs in bone as in other connective tissues. However, the degree of pigment accumulation is much lower than in cartilage, probably because cartilage is a stable tissue, which remains exposed to the dangerous effects of ochronosis over long periods, whereas bone is continuously remodelled and renewed. Moreover, pigment deposition in bone occurs in a calcified matrix, whose collagen fibrils are encrusted with calcium ions and whose physiological properties (stiffness, elasticity, plasticity) are therefore unlikely to be modified. Pigment accumulation in osteocytes, and their consequent degeneration and death, might represent a severe cause of bone failure, because these cells are indispensable for physiological bone activity [3]. However, the low numbers of pigmented osteocytes suggest that their alteration or death has no significant repercussions for the bone tissue as a whole.

Acknowledgements This investigation was supported by grants from the Italian Ministry of University and Scientific and Technological Research (M.U.R.S.T.), the National Research Council (C.N.R.), and ‘La Sapienza’ University of Rome.

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