CASE REPORT

The Distribution of Ochronosis in Osteoarthritic Articular Cartilage in a Knee

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joint arthroplasty. The questions are (1) which zones in the articular cartilage accumulate ochronotic staining and (2) are other associated tissues, i.e., osteophytes, pigmented?

Introduction

Alkaptonuria (AKU) is a rare, genetic, autosomal-recessive disorder characterized by a defect in the tyrosine metabolic pathway [2, 6, 9, 10]. AKU patients are deficient for a single enzyme, homogentisate 1,2-dioxygenase (HGD) [2], which is responsible for the breakdown of homogentisic acid (HGA) into 4-maleylacetoacetic acid [6, 9, 10]. AKU has a prevalence lower than 1:250,000 in most populations [9, 10]. Affected individuals excrete HGA in the urine, causing a darkened color when the urine is oxidized [9, 10]. Accumulation of HGA in tissues causes ochronosis, a condition in which there is a deposition of brown-black pigment in connective tissues such as cartilage, skin, and sclerae [6, 8–10]. The mechanism of ochronotic deposition begins with polyphenol oxidases in skin and cartilage that oxidize HGA to form benzoquinones. The benzoquinones then polymerize into “melanin-like” compounds, giving connective tissue and skin the brown-black pigment [5]. One of the tissues exhibiting ochronosis is articular cartilage. This tissue becomes weak and brittle, developing cracks and chips and leading to chronic inflammation and degeneration [5].

Our objective is to report a case of ochronosis and describe in detail the pigment distribution in articular cartilage tissue from a 73-year-old patient who underwent total

Case Report

The patient is a 73-year-old Chinese female who presented to the hospital with back stiffness and marked left knee pain. The patient underwent a total knee replacement of the left knee. Samples were taken and processed for histology by decalcification and paraffin embedding. Seven-micrometer sections were cut and alternate slides were either (1) left unstained or (2) stained with either hematoxylin and eosin (H&E) or toluidine blue. Toluidine blue was chosen for its ability to increase the sharpness of histology slide images. Prepared slides were examined with a Nikon Eclipse 90i microscope, and pictures were taken using NIS Element Software.

Macroscopic examination of tissue samples revealed brown pigmentation across the articular cartilage consistent with the diagnosis of ochronosis. Figure 1 demonstrates, at microscopic level and low power, the distribution of pigment in articular cartilage. In this case, the transitional and radial zones displayed heavy ochronotic pigmentation, while the superficial articular cartilage and osteophyte did not (Fig. 1).

Microscopic examination in the absence of any histological stain revealed that pigmentation was present primarily in the radial zone of the samples (Fig. 2a). Pigmentation appeared intracellular within chondrocytes and intercellular within the extracellular matrix (Fig. 2b). The superficial zone on the articular surface and the calcified zone on the subchondral surface, along with the subchondral bone, appeared free of pigmentation. However, light brown pigmentation can be seen pericellular for some chondrocytes in the calcified zone (Fig. 2b).

Analysis with hematoxylin and eosin and toluidine blue revealed similar results to the unstained sections regarding the distribution of ochronotic pigmentation. In H&E-stained

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sections, the ochronotic pigmentation remained brown, while the unaffected tissue stained the standard pink and purple. The heaviest ochonotic deposits were located in the radial zone around the territorial matrix of the lacunae (Fig. 3). The radial zone displayed blanket brown pigmentation of ochronosis in the matrix, while the superficial and calcified zones showed pigmentation normal for H&E staining (Fig. 3a). The extracellular matrix of the superficial zone stained pink, while the nuclei and cell-dense regions stained darker pink or purple (Fig. 2b). The matrix of the calcified zone appeared a light pink, as did the subchondral bone matrix (Fig. 3c). The radial zone exhibited the expected numerous isogenous nests of chondrocytes (Fig. 2b), but the calcified cartilage zone also contained a few isolated isogenous nests (Fig. 2c).

Toluidine blue sections were consistent with H&E sections. The brown homogentisic acid combined with toluidine blue produced a dark green, while unaffected tissue stained blue to purple (Fig. 4). Pigmentation was heaviest in the radial zone, being present throughout the extracellular matrix (Fig. 4a). The territorial matrix of the lacunae did not display dense pigmentation as under H&E. The superficial zone was absent from this section, and heavy proliferation can be seen in the transitional zone (Fig. 4b). The heaviest pigmentation appeared intercellular in the extracellular matrix in the radial zone (Fig. 4a, c). Note the difference in matrix between blue-stained calcified cartilage and clear to light-blue subchondral bone (Fig. 4c).

Some samples included what appeared to be a forming osteophyte lateral to the affected articular cartilage. Osteophytes are signs of osteoarthritis that develop from proliferating mesenchymal stem cells as bony outgrowths at the margins of joints. Histological samples of this outgrowth displayed no ochronotic pigment. The matrix within the outgrowth appeared pink and purple under H&E (Fig. 5a) and blue to purple under toluidine blue (Fig. 5b). No endochondral ossification was observed in the sample available, although

Fig. 1. Low power photomicrograph of articular cartilage stained with H&E displaying all layers of articular cartilage: superficial zone (sz), transitional zone (tz), radial zone (rz), calcified zone (cz) as well as subchondral bone (sb) and forming osteophyte (o).

there is histologic bone at the lateral periphery of the forming osteophyte contiguous with the metachromatic staining seen with toluidine blue staining (Fig. 5b).

Discussion

The samples available for this study contained sections of intact articular cartilage, where the entire component of articular cartilage (the superficial zone through the calcified cartilage) remained in place. This allowed observations of all the layers of articular cartilage. Articular cartilage consists of four horizontal zones [4, 12]. The superficial zone, adjacent to the surface, is the thinnest zone. It consists of numerous flattened chondrocytes arranged tangentially to the surface. The next zone, the transitional zone, has increasing proteoglycan content and contains round chondrocytes randomly organized within the matrix [4]. The largest section, the radial zone, displays small round chondrocytes in short

Fig. 2. Unstained photomicrographs of articular cartilage in a sample of a patient with ochronosis. **a** Overview of full-depth medial tibial plateau, showing brown ochronotic pigment. superficial zone (sz), transitional zone (tz), radial zone (rz), calcified zone (cz). Bar=500 μm. **b** Higher magnification view of radial zone (rz) and calcified zone (cz) of articular cartilage. Arrow points to isogenous nests with intracellular pigmentation. Bar=100 μm.

Fig. 3. Photomicrographs of ochronotic articular cartilage stained with H&E. **a** Overview of full-depth articular cartilage, showing brown ochronotic pigmentation. superficial zone (sz), transitional zone (tz), radial zone (rz), calcified zone (cz). Intervening space is an artifact (a) of fixation. Bar=500 µm. **b** Higher magnification view of upper right area in (a) showing the superficial zone and transitional zone. Bar=100 µm. **c** Higher magnification of lower left area in (a) showing the radial zone, calcified zone, and subchondral bone (sb). Arrows point to isogenous nests with intracellular pigmentation. Bar=100 µm.

columns perpendicular to the surface and the lowest water concentration. Lastly, the calcified zone, divided from the radial zone by a heavily calcified, basophilic line called the tidemark, contains small chondrocytes within a calcified matrix, and borders the subchondral bone [12]. The calcified zone contains collagen fibrils arranged perpendicular to the surface and lacks proteoglycans [4]. Proliferation of chondrocytes within lacunae pockets allows interstitial growth. This takes place in the zones above the tidemark [12].

Samples from this patient contain very advanced ochronosis, in which pigmentation is present throughout the radial zone and its chondrocytes (Figs. 2 and 3). Intracellular pigmentation in chondrocytes as well as intercellular pigmentation in the extracellular matrix was observed (Figs. 3 and 4). However, the superficial zone was completely devoid of ochronotic staining, and the calcified cartilage exhibited only a few instances of staining in the pericellular region, with the matrix devoid of pigmentation (Figs. 3 and 4). These findings provide insight into the accumulation of

Fig. 4. Photomicrographs of ochronotic articular cartilage stained with toluidine blue. **a** Overview of full-depth articular cartilage, showing green ochronotic pigmentation. transitional zone (tz), radial zone (rz), calcified zone (cz), subchondral bone (sb). Note the superficial zone is absent. Bar=500 µm. **b** Higher magnification of the radial zone and transitional zone. Bar=100 µm. **c** Higher magnification of lower radial zone, calcified zone, and subchondral bone. Bar=100 µm.

Fig. 5. Photomicrographs of ochronotic articular cartilage with an apparent osteophyte under H&E staining (**a**) and toluidine blue staining (**b**). articular cartilage (AC), osteophyte (O), bone within osteophyte (B). This cartilage is at the edge of the tibial plateau and contiguous with that seen in Figs. 1, 2, and 3. Bar=500 µm.

the polymers of benzoquinone, which are the pigmentation product of homogentisic acid.

Homogentisic acid (HGA) is produced by the catabolism of tyrosine and phenylalanine within tissues [6, 9]. Tyrosine and phenylalanine are obtained by the enzymatic degradation of proteins. Since AKU patients lack the gene for homogentisate 1,2 dioxygenase to metabolize HGA, the level is elevated in plasma [9]. HGA is oxidized to benzoquinone, which then polymerizes to form the melanin-like molecules with brown pigmentation. Diffusion from the synovial fluid could account for increased HGA levels in cartilage. However, there is evidence that chondrocytes normally express the enzyme homogentisate 1,2 dioxygenase [7, 11]. Chondrocytes also contain polyphenol oxidases, which catalyze the oxidation of HGA into pigment [19]. Chondrocytes obtained from AKU patients produce pigment in cell culture [7, 13, 17], indicating that at least some of the HGA, and pigment, in ochronotic cartilage originates within the tissue.

That the pigment originates within cartilage is supported by the observation that pigmentation in the early stage of ochronosis is associated with individual chondrocytes, both intracellular and pericellular in the chondrocyte lacunae and territorial matrix [14]. Blanket pigmentation in the radial and transitional zone occurred in the later stages of ochronosis [14, 16]. In a murine model of AKU, Taylor and colleagues observed ochronotic pigmentation in the radial chondrocytes, as well as intra- and pericellular pigmentation of more superficial chondrocytes [15]. Our results are consistent with these previous results, as we also observed intracellular pigmentation (Fig. 3) and blanket pigmentation in the transitional and radial zones (Figs. 1, 2, 3, and 4). The pattern of staining within the zones of the articular cartilage is also consistent with HGA production and deposition of pigment by chondrocytes. Ochrotonic staining was not observed in the superficial zone or the calcified zone (Figs. 1, 2, 3, and 4).

Cells that are metabolically active, with an active turnover of proteins, will produce quantities of homogentisic acid. The majority of ochronosis arises from the spread of the polymers to established matrix, not by secretion of pigmented matrix [4], supported by the observation of intracellular pigmentation (Fig. 3) [7, 14, 15, 17]. Ochrotonosis can cause many changes in connective tissues. Highly vascularized tissues will clear the homogentisic acid produced by cells prior to polymerization, thus preventing deposition of those polymers in the matrix. Chondrocytes in the superficial, transitional, and radial zones are metabolically active, participating in the maintenance of the extracellular matrix. Thus, chondrocytes in these zones can reasonably be expected to catabolize proteins and produce homogentisic acid. However, cartilage is avascular and receives its nutrients by diffusion from the synovial fluid [1]. Water moves from articular cartilage to the synovial fluid and back under mechanical pressure. This movement is greatest through the superficial zone and would tend to clear homogentisic acid from that zone, while the movement is less for the transitional and radial zones [11]. Homogentisic acid, whether endogenously produced or exogenously diffused, would accumulate within the matrix of the transitional and radial zones, where it can oxidize to benzoquinone, polymerize, and bind to extracellular matrix molecules [5]. Thus, the movement of water could explain the lack of ochrotonic staining in the superficial zone and its presence in the transitional and radial zones, resulting in the observed blanket staining of those zones.

Chondrocytes in the calcified zone have little to no metabolic activity [4] nor does the calcified matrix experience a high rate of turnover. Thus, chondrocytes in the calcified zone would produce only small amounts of homogentisic acid, which is shown in this sample by intracellular pigmentation in a few chondrocytes in that zone (Fig. 2b).

Bone undergoes remodeling, and the resorption phase would remove the bone matrix and any homogentisic acid

or polymers within it. This explains the lack of pigmentation in the subchondral bone.

Of particular interest in our case was the lack of ochronotic pigment in a newly formed osteophyte, a finding we believe has not been previously reported. A recent paper by Yamada et al., however, does show gross lack of ochronotic staining in an osteophyte, although it is not noted [18]. The apparent forming osteophyte is in the formation phase of bone, with the synthesis of new organic matrix, not the catabolism of existing proteins. The tissue is also relatively recent, which means that there has been little time for the accumulation of homogentisic acid formation produced by the cells in the matrix, its oxidation to benzoquinone, or the formation of polymers, which provide an explanation of the lack of pigmentation.

There are other potential explanations of the lack of ochronotic pigment in the osteophyte. Osteophyte cartilage has been shown to be different than normal articular cartilage. Osteophytic and articular chondrocytes significantly differ in their gene expression. Articular cartilage expresses inhibitors of the BMP and WNT pathways, which may serve to stabilize permanent chondrocyte phenotype and thus prevent terminal differentiation [3]. In contrast, osteophytic chondrocytes express genes involved in the endochondral ossification process [3]. In endochondral ossification, the chondrocytes and matrix are resorbed and replaced by bone. Any ochronotic polymers of benzoquinone will be removed during resorption.

Our study of the ochronotic pigment distribution in a degenerative knee indicated preferential staining in the radial layers of articular cartilage and a lack of staining in the superficial zones and the osteophyte. This suggests that pigment deposits preferentially in high metabolic tissue and less so in low turnover tissue and newly formed tissue.

Disclosures

Conflict of Interest: Vincent W. Vigorita, Paul D. Marino, BA, and Paul A. Lucas, PhD received institutional research grant support from the New York Community Trust for the study.

Human/Animal Rights: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5).

Informed Consent: N/A

Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

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