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A novel *ex vivo* organotypic culture model of alkaptonuria-ochronosis

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ABSTRACT

Objective. *Alkaptonuria (AKU) is an orphan disease that has an estimated prevalence of 0.3/100,000. The disease is caused by the lack of activity of homogentisic acid oxidase (HGO), an enzyme involved in tyrosine and phenylalanine metabolism. To date, there is only one drug, the nitisinone, with orphan designation authorised by both Food and Drug Administration (FDA) and European Medical Agency (EMA) for AKU. A clinical trial on AKU patients using nitisinone has recently been completed but it needs further investigation for long-term therapy. In recent years our group has developed a series of AKU in vitro models using cell lines, primary chondrocytes and human plasma in order to test the efficacy of new substances, mainly antioxidant compounds, for AKU therapy. Herein, we report the optimisation of an ex vivo reproducible culture method exploiting cartilage slices in order to investigate the deposition of ochronotic pigment in this kind of connective tissue.*

Methods. *Human normal cartilage slices, obtained after surgery for prosthesis replacement, were cultured for several days in the presence of a sublethal concentration of homogentisic acid (HGA).*

Results. *After two months of incubation with HGA, the peculiar melanin-like ochronotic pigmentation can be observed into the cartilage tissue.*

Conclusions. *This novel organotypic ex vivo model could be extremely useful to investigate the efficacy of substances able to ameliorate the conditions of AKU patients. Moreover, it could be used for genetic and proteomic investigations to better define AKU pathophysiology.*

Introduction

Alkaptonuria (AKU) is a rare "inborn" disease (1) developed from mutations in the *hgd* gene that lead to the lack of homogentisic acid oxidase (HGO) activity. HGO is an enzyme involved in phenylalanine and tyrosine metabolism. The guilty discriminator of AKU clinical manifestations is homogentisic acid (HGA) that, under its oxidised form, develops melanin-like polymers. Afterwards, these polymers accumulate in connective tissues causing ochronosis. HGO has a strong tissue-specific expression in liver, kidney, small intestine, colon, and prostate (2). However, it has been reported that cartilage is one of the most ochronosis-affected tissues, even if the expression of *hgd* gene in chondrocytes has not been demonstrated yet .

To date, there is no effective therapy for AKU. Medical treatment is supportive and is directed toward improving the patient's quality of life. The herbicide nitisinone (Orfadin) is the only drug with orphan designation for AKU by FDA (14) and EMA (15). However, the results of the recently completed 3-year phase II clinical trial with nitisinone suggest the need for further investigation before its approval (3).

Recently, we have proposed three *in vitro* models of AKU-ochronosis based on HGA-treated chondrocytic and osteoblastic cell lines (4, 5), primary chondrocytes cultures (6) and human plasma (7). These models were successfully adopted to evaluate the efficacy of different antioxidant compounds in the reduction of ochronosis pigmentation. The observed results were suggestive of a potential efficacy of the tested compounds in clinical therapy.

The development and optimisation of AKU *in vitro* models have advanced our understanding of AKU. However, the methods have substantial limitations in evaluating the deposition of ochronotic pigment in a complex and three-dimensional environment such as the cartilageneous matrix. For this reason, newer, more robust and innovative models need to be developed. With this in mind, we have investigated whether an *ex vivo* organotypic culture based on cartilaginous tissue slices could be successfully employed. Hence, we set up an *ex vivo* model using human articular cartilage slices challenged over a long period with a HGA concentration in the same range of the human plasma HGA levels observed in AKU patients (4-7). The special advantage of our new approach is the ability to maintain both organ and cellular architecture leading to the formation of ochronotic pigment under physiological conditions as in an *in vivo* model.

Materials and methods

All reagents for cell culture were purchased from Invitrogen, San Giuliano Milanese (MI), Italy. Human articular cartilage was obtained from the femoral heads of patients who had undergone surgery for total hip replacement following a fracture. The study received approval from the Local Ethics Committee. Immediately after surgery, macroscopically cartilage was cut aseptically and minced in slices using a sterile scalpel, obtaining as far as possible slices of the same thickness (about 0.5mm). Healthy cartilage slices have been obtained by a single tangential cut in the median region of articular femoral heads. The fragments were washed in Dulbecco's modified Eagle's medium (DMEM) containing 2% penicillin/streptomycin (P/S) solution and 0.2% amphoterycin B.

Afterwards, human normal cartilage slices were transferred to a 6 well/plate, one slice for each well. A series of samples was incubated with DMEM+10% foetal calf serum (FCS) +2% P/S. These were the controls. Another series of samples was incubated with DMEM+10%FCS+2% P/S charged with 0.33 mM of HGA purchased from Sigma-Aldrich, Milan Italy (6).

The medium was refreshed every two days. The cartilage fragments were monitored over a 2-month culture period (Fig. 1).

The cartilage cultures were performed in parallel on three different samples from two different donors.

Results

We present a novel cartilage model of alkaptonuria. Our methodology takes advantage of the rapid sectioning of cartilage from femoral head immediately after surgery for prosthesis replacement. After two days of treatment with HGA excess, the cultural supernatant of the treated cartilage cultures started to show a darker colouration in comparison to the supernatant of the control cultures, while such darkening was not observed in incubating for the same period HGA to the cultural medium in the absence of cartilage slices (data not shown). This phenomenon resembled the urine darkening reported in AKU patients due to oxidation of the excreted

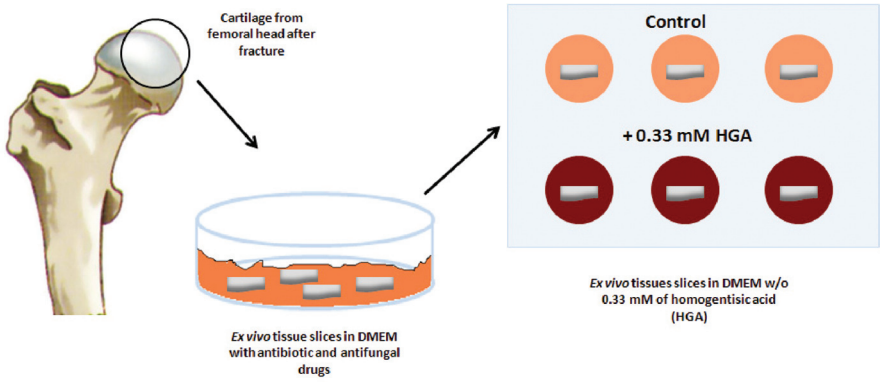

Fig. 1. Schematic representation of the methodology for the setting up of the cartilage organotypic *ex vivo* model of alkaptonuria-ochronosis.

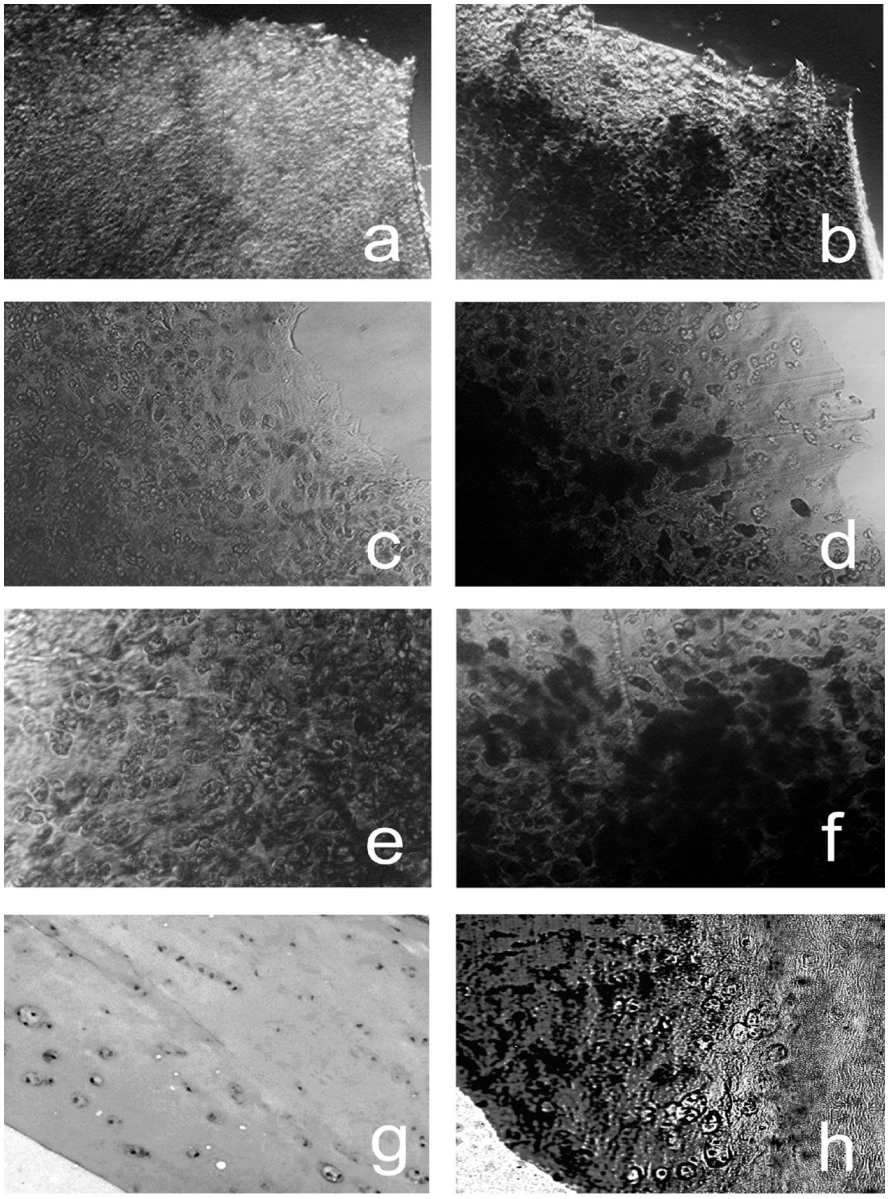

Fig. 2. Microscopy of the AKU cartilage model. Images on the left column: (**a, c, e, g**), cartilage slices after a 2-month culture period in DMEM without HGA (Controls). Images on the right column: (**b, d, f**), cartilage slices after 2 months of culture in DMEM with addition of HGA (0.33 mM); (**h**), ochronotic cartilage from an AKU patient. Original magnification: 4x (a-b), 10x (c-d), 20x (e-f), 10x (g-h).

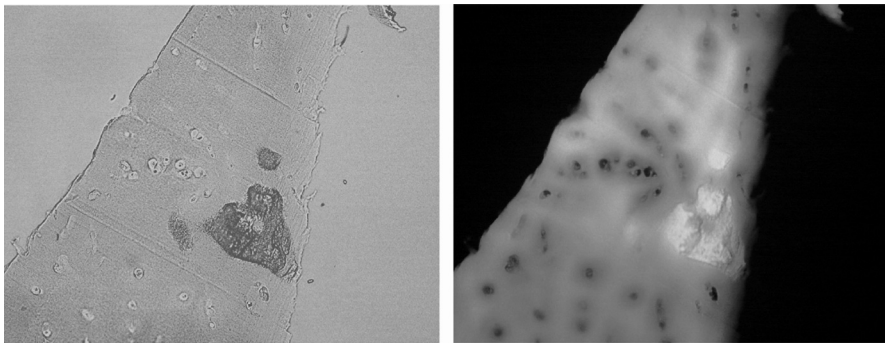

Fig. 3. Melanin-like pigmented areas present in AKU cartilage model were evaluated for their intrinsic fluorescence properties irradiating specimens under UV light (left). These areas perfectly co-localised with ochronotic pigment (right).

HGA under aerobic conditions. Tissue examination under optical microscope confirmed, in both normal and treated cartilage, morphological integrity of tissues, defined as preservation of general architecture. The medium of every sample was refreshed every two days for a month when we started to observe the first formation of ochronotic pigment.

The HGA deposits varied in location and distribution along the cartilage. Indeed, some areas showed only minute granules whilst other showed more severe advanced forms of pigmentation. In other regions no pigmentation occurred. The smaller granules likely represent nucleation points for the polymerisation process that leads to the formation of larger ochronotic deposits as already hypothesized (8).

Importantly, under the conditions adopted in our model, we observed pigment depositions similar to those seen in the cartilage of ochronotic AKU patients. The pigment deposition was morphologically mainly localised in and around the lacunae space (Fig. 2). In such a way, it is reasonable that chondrocytes in the lacunae could be seriously stressed by the extensive pigmentation which could obstruct the diffusion of nutrients. Our results again confirmed that the presence of HGA cannot be the only factor in pigmentation, since other local factors could promote or inhibit nucleation and deposition of ochronotic pigmentation (8, 9).

Moreover, the presence of melanin-like pigmentation is strengthened by the evidence of intense fluorescence after a brief UV illumination of pigment deposition (Fig. 3).

Discussion

The main deficiency in AKU research has so far been the lack of suitable models to study the mechanisms involved in AKU pathogenesis. For example, an animal model that shows ochronosis (10) has not been developed yet, although recent progress has been reported (11). Moreover, *in vitro* models, which were developed many years ago, have never been optimised (12, 13).

For this reason, our research group recently developed a series of novel AKU models (4-7). Since the pathogenesis of the disease has not been studied for years, our first aim was to obtain a series of methodologies that could recreate *in vitro* the conditions of AKU in patients. Our first *in vitro* AKU model was obtained by exploiting cell lines treated with an excess of HGA (ranging HGA plasma levels in AKU patients) in order to develop intra- and extra-cellular ochronotic pigmentation (4, 5). A second *in vitro* model was based on primary cells (chondrocytes, osteoblasts and synoviocytes) from non-diseased patients (non-AKU cells), treated with an excess of HGA (6). Another *in vitro* model was set up using human plasma challenged with the same HGA excess, to produce large amounts of ochronotic pigment (7). Each model has specific features that provided new insights in AKU research. On the other hand, no model showed limitations for an investigation into deposition of ochronotic pigmentation.

For this reason, we set up a novel *ex vivo* organotypic culture model that closely mimics the disease, as an implementation of the previous *in vitro* AKU models: it offers the same advantages but within a more complex tissue system based on human normal cartilage cultured in HGA-supplemented medium. The AKU cartilage model produced HGA-induced melanin like pigmentation and can be used as a source of ochronotic pigment as well as a tissue system to assay drugs.

Moreover, the set up of this model is crucial in order to study the diffusion of HGA through three-dimensional tissue and to highlight the mechanism of accumulation of HGA in avascular tissue such as cartilage.

Furthermore, our study provides a novel and optimised methodology that could be reproducible in every laboratory.

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