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Ochronosis in a murine model of alkaptonuria is synonymous to that in the human condition

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SUMMARY

Objective: Alkaptonuria (AKU) is a rare genetic disease which results in severe early onset osteoarthropathy. It has recently been shown that the subchondral interface is of key significance in disease pathogenesis. Human surgical tissues are often beyond this initial stage and there is no published murine model of pathogenesis, to study the natural history of the disease. The murine genotype exists but it has been reported not to demonstrate ochronotic osteoarthropathy consistent with the human disease. Recent anecdotal evidence of macroscopic renal ochronosis in a mouse model of tyrosinaemia led us to perform histological analysis of tissues of these mice that are known to be affected in human AKU.
Design: The homogentisate 1,2-dioxygenase $Hgd^{+/-}Fah^{-/-}$ mouse can model either hereditary tyrosinaemia type I (HT1) or AKU depending on selection conditions. Mice having undergone Hgd reversion were sacrificed at various time points, and their tissues taken for histological analysis. Sections were stained with haematoxylin eosin (H&E) and Schmorl's reagent.
Results: Early time point observations at 8 months showed no sign of macroscopic ochronosis of tissues. Macroscopic examination at 13 months revealed ochronosis of the kidneys. Microscopic analysis of the kidneys revealed large pigmented nodules displaying distinct ochre colouration. Close microscopic examination of the distal femur and proximal fibula at the subchondral junctions revealed the presence of numerous pigmented chondrocytes.
Conclusions: Here we present the first data showing ochronosis of tissues in a murine model of AKU. These preliminary histological observations provide a stimulus for further studies into the natural history of the disease to provide a greater understanding of this class of arthropathy.

Introduction

Alkaptonuria (AKU) is a rare autosomal recessive condition that is caused by a single enzyme deficiency in the tyrosine metabolic pathway[1]. The absent enzyme is homogentisate 1,2-dioxygenase (HGD), which is responsible for the conversion of homogentisic acid (HGA) to 4-maleylacetoacetic acid (MAA)[2]. Enzyme loss results in accumulation of HGA in both tissues and circulating blood[1]. HGA shows a particularly high affinity for collagenous tissues; initial deposits are associated with the periodicity of collagen fibres[3]. Between the third and fourth decade of life, accumulation of HGA polymers begins to manifest in joint disorders, culminating in devastating arthropathy. Furthermore, HGA-associated pigment known as ochronosis has detrimental effects on other collagenous matrices in the body[4,5]. Ochronosis of cartilage was recently shown to be initiated in individual chondrocytes and their territorial matrix, before progressing to a more widespread pigmentation of the cartilage matrix[6]. Elucidation of the mechanism by which ochronosis begins and progresses will provide new targets for intervention. It has been suggested that 2-[2-nitro-4-(trifluoromethyl)benzoyl]cyclohexane-1,3-dione (NTBC), the therapeutic agent used in treating a related tyrosine catabolic disorder hereditary tyrosinaemia type I (HT1), may prove beneficial in treating AKU by blocking the production of HGA in the breakdown of tyrosine[7] (Fig. 1). The efficacy and safety associated with the use of this drug to treat AKU has yet to be determined, but testing would be facilitated by access to a mouse model of joint pathology.

A murine model of AKU was generated in 1994 by ENU-induced mutagenesis[8]. The AKU mutation was then backcrossed onto both

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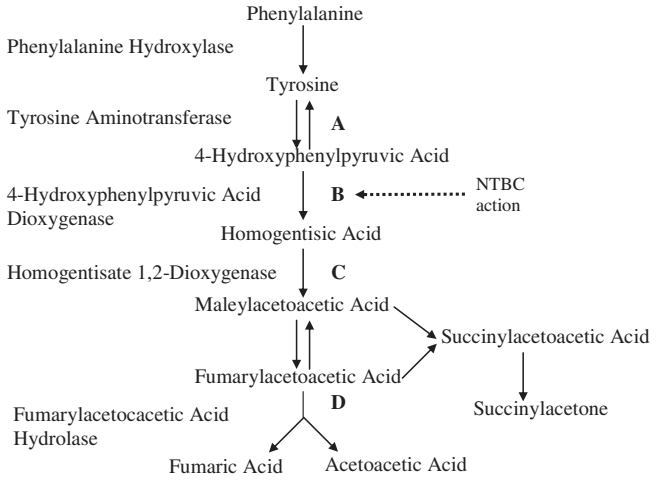

Fig. 1. Diagram of the phenylalanine and tyrosine metabolic pathway highlighting enzymes and associated defects. A = Tyrosinemia type II, B = Tyrosinemia type III, C = AKU, D = Tyrosinemia type I. NTBC is shown at its site of action.

the BALB/cByJ and the C57/BL/6J backgrounds. These animals were previously thought not to exhibit ochronosis, despite excreting sufficient HGA to cause darkening of urine[9]. The mice have truncated HGD protein resulting from a splice mutation[8].

The related tyrosine disorder HT1 is caused by a deficiency of (*Fah*) – the terminal enzyme in the pathway – that causes progressive liver disease and renal tubular dysfunction[10]. In both HT1 patients and $Fah^{-/-}$ mice, toxic metabolites like fumarylacetoacetate (FAA) accumulate causing death in a cell-autonomous manner. Treatment with NTBC, an inhibitor that blocks upstream of FAH, prevents metabolite accumulation and rescues the phenotype[11]. $Hgd^{-/-}$ and FAA hydrolase (*Fah*) deficient mice have been crossed for several generations to produce $Hgd^{-/-}Fah^{-/-}$ mice[12]. Interestingly, some mice with the intermediate genotype $Hgd^{+/-}Fah^{-/-}$, were resistant to liver failure characteristic of HT1 when withdrawn from NTBC. Histological examination showed healthy liver nodules in these mice, suggesting reversion had occurred at the *Hgd* locus. The reversion is thought to arise as a response to the production of reactive compounds in the liver and kidneys, namely FAA, and other subsequent spontaneously formed derivatives. Individual hepatocytes that revert to the double knockout genotype have a selective advantage within the liver as they do not produce the toxic derivatives[12]. The accumulation of such hepatotoxic compounds can be prevented by NTBC administration. $Hgd^{+/-}Fah^{-/-}$ neonates must nurse from a mother on NTBC as neonates are acutely sensitive to HT1-related hepatotoxin accumulation during this developmental window and will die from acute liver failure within 24 h without NTBC from the breast milk. However, when $Hgd^{+/-}Fah^{-/-}$ adult mice are withdrawn from NTBC, a small percentage will undergo a loss of heterozygosity (LOH) event at the *Hgd* locus generating an $Hgd^{-/-}Fah^{-/-}$ hepatocyte that is resistant to effects of the FAH deficiency. These reverted hepatocytes now have a selective advantage and can clonally expand to repopulate the liver. As a consequence, the block to HGD leads to an accumulation of HGA in the liver and blood stream.

Other models of ochronosis have been reported, but are generally not useful or viable for experimental studies[13,14]. Following anecdotal descriptions of ochronosis in $Hgd^{+/-}Fah^{-/-}$ mice withdrawn from NTBC, we undertook both a macro- and microscopic study of their tissues. Here we describe the first detection of ochronosis in a murine model of AKU and similarities between the initial stages of pigmentation in the murine model and those of the human condition. We expect that this murine model will prove beneficial for screening therapeutic agents for treating the condition.

Materials and methods

Mouse strains and animal husbandry

$Hgd^{-/-}$ *and* $Fah^{-/-}$ mice were bred to generate $Hgd^{+/-}Fah^{-/-}$ mice. The *Hgd* mutation was generated by an ENU-mutagenesis screen[8], while the *Fah* mutation was generated by neo-cassette insertion into exon 5[15]. The resultant $Hgd^{+/-}Fah^{-/-}$ can model either HT1 or AKU depending on selection conditions from the time spent off NTBC. $Hgd^{+/-}Fah^{-/-}$ neonates will die from acute HT1–related liver failure if NTBC is not continually administered. NTBC treatment at 4-mg/L in the drinking water prevents hepatorenal injury and rescues the phenotype. Short-term NTBC withdrawal allows modelling of HT1 as mice rapidly develop hepatorenal disease over the course of several weeks and die of end-stage liver disease in 6–8 weeks[10]. Long-term NTBC withdrawal can stimulate LOH at the *Hgd* locus resulting in mice with an $Hgd^{-/-}Fah^{-/-}$ genotype. These mice model AKU and are protected from HT1-related damage from the upstream *Hgd* mutation[9]. Depending on the selection conditions desired, mice were maintained on either 4-mg/L NTBC in the drinking water or water with no added drug, and fed irradiated high-fat low-protein mouse chow (Lab Diet Cat#Picolab 5LJ5) *ad libitum* to decrease flux through the tyrosine pathway. The Institutional Animal Care and Use Committee of Oregon Health & Science University approved all procedures and mouse experiments.

Histology

$Hgd^{+/-}$ $Fah^{-/-}$ mice having undergone *Hgd* reversion were sacrificed at various time points; one mouse (male) at 8 months and six mice (four males and two females) at 13 months, and their tissues taken for analysis. Kidneys were sectioned through the midline, dividing into an anterior and posterior half prior to processing. Tissue samples were washed in phosphate buffer saline (PBS) and then fixed in 10% phosphate buffered formol saline (PBFS). Mineralized tissues were decalcified with 12% ethylenediaminetetraacetic acid (EDTA) in 10% formalin. Following fixation and/or decalcification, tissues were routinely processed for histology and paraffin embedded. Five-micrometre sections were cut and mounted on glass slides, knees were sectioned transversely from lateral to medial. Sections were stained with either haematoxylin eosin (H&E) (every third section) or Schmorl's reagent (every fourth section) which we have previously shown to be a sensitive stain for tissue ochronosis[16]. Stained sections were dehydrated and mounted in Dibutyl phthalate in Xylene (DPX) (Sigma, UK).

Results

Macroscopic findings

Early time point observations of survivors at 8 months off NTBC showed no sign of macroscopically visible ochronosis to the skin, eyes, ears, femur, knee joint, vertebrae or various cartilages. An examination of the liver showed a mosaic pattern of nodules of relatively normal tissue among areas of damage. Histological analysis of early time point animals was not undertaken.

Interestingly, macroscopic examination of the liver at 13 months off NTBC revealed yellow-brown discolouration with signs of