

Original article

Development of an *in vitro* model to investigate joint ochronosis in alkaptonuriaLaura Tinti^{1,2}, Adam M. Taylor², Annalisa Santucci¹, Brenda Wlodarski², Peter J. Wilson², Jonathan C. Jarvis², William D. Fraser³, John S. Davidson⁴, Lakshminarayan R. Ranganath³ and James A. Gallagher²

Abstract

Objectives. Alkaptonuria (AKU) is a genetic disorder caused by lack of the enzyme responsible for breaking down homogentisic acid (HGA), an intermediate in tyrosine metabolism. HGA is deposited as a polymer, termed ochronotic pigment, in collagenous tissues. Pigmentation is progressive over many years, leading to CTDs including severe arthropathies. To investigate the mechanism of pigmentation and to determine how it leads to arthropathy, we aimed to develop an *in vitro* model of ochronosis.

Methods. Osteosarcoma cell lines MG63, SaOS-2 and TE85 were cultured in medium containing HGA from 0.1 μ M to 1 mM. Cultures were examined by light microscopy and transmission electron microscopy, and Schmorl's stain was used to detect pigment deposits *in vitro*, following the observation that this stain identifies ochronotic pigment in AKU tissues. The effects of HGA on cell growth and collagen synthesis were also determined.

Results. There was a dose-related deposition of pigment in cells and associated matrix from 33 μ M to 0.33 mM HGA. Pigmentation *in vitro* was much more rapid than *in vivo*, indicating that protective mechanisms exist in tissues *in situ*. Pigment deposition was dependent on the presence of cells and was observed at HGA concentrations that were not toxic. There was an inhibition of cell growth and a stimulation of type I collagen synthesis up to 0.33 mM HGA, but severe cell toxicity at 1 mM HGA.

Conclusion. We have developed an *in vitro* model of ochronosis that should contribute to understanding joint destruction in AKU and to the aetiology of OA.

Key words: Alkaptonuria, Ochronosis, Arthropathy, Osteoarthritis, Homogentisic acid, *In vitro* model.

Introduction

Alkaptonuria (AKU) is a rare inborn error of metabolism with an incidence of approximately 1 in 250 000 people. The highest prevalence is in populations of some parts of Eastern Europe, where it occurs at 1 case per 25 000 people [1]. AKU is caused by deficiency of homogentisate 1,2-dioxygenase (HGD) activity in the liver [2], since the

gene for HGD is mutated in AKU patients [3, 4]. The lack of HGD activity leads to elevated levels of homogentisic acid (HGA), which is an intermediary product of tyrosine metabolism. Excess HGA is excreted daily in the urine, giving a black colour after oxidation [5]. This colouration is the first and best-known manifestation of the disease. Some evidence suggests that it is not HGA itself but a by-product of its oxidation, a benzoquinone acetic acid, that can polymerize to a melanin-like pigment by unknown mechanisms and accumulates both inter- and intracellularly in connective tissue, most commonly the joints, cardiovascular system, kidney, skin and glands [2, 4, 6, 7, 8], a pathological condition known as ochronosis. The major clinical manifestations of AKU usually become evident in the fourth or fifth decade of life and are related to deposition of ochronotic pigment in affected tissues. One of the most important complications of AKU is ochronotic arthropathy caused by polymer deposition in cartilage.

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Several reports suggest that the first site of pigmentation is the chondrocyte but others have noted that pigment deposition occurs mainly in the ECM [9]. The reason for the affinity of ochronotic pigment to connective tissue is not known, but the pigment seems to have a particular affinity for fibrillar collagens that are surrounded by mucopolysaccharide ground substance.

To date, there is still no effective treatment for AKU. Dietary modifications to restrict intake of tyrosine and phenylalanine have been met with limited success owing to the difficulty in maintaining such a restrictive diet for the whole of a person's life. There is some indication that ascorbic acid might have a protective effect, but results have been variable [10, 11]. More recently, nitisinone, a potent inhibitor of the enzyme responsible for converting hydroxyphenylpyruvate to HGA, has also been evaluated [12, 13]. Nitisinone has been shown to significantly lower the urinary excretion of HGA in both murine models and humans but there are no clinical trial data suggesting efficacy in long-term therapy [14].

There are major limitations in studying the ochronosis of AKU. It is a rare disease and pigment deposition in AKU subjects occurs over a long time course. Although HGD-deficient mice exist, they do not normally develop ochronosis. Hence, to investigate the mechanism of formation of ochronotic pigment and to determine how the deposition of pigment in collagenous matrix leads to arthropathy, we have developed an *in vitro* model of ochronosis using osteosarcoma cell lines cultured for extended periods in medium containing HGA, at concentrations that include the range observed in the plasma of AKU patients *in vivo*. This *in vitro* model could contribute to a general understanding of the mechanism of ochronotic pigment deposition, the aetiology of the resulting arthropathies and to the development of strategies for preventing ochronosis.

Materials and methods

Materials

All reagents including HGA were obtained from Sigma-Aldrich (Dorset UK) unless otherwise stated.

Tissue histology

Tissues were obtained from a patient with AKU at the time of surgery with informed patient consent and with ethical approval from Liverpool Research Ethics Committee. Tissues were fixed in 10% phosphate buffered formal saline (PBFS), processed by routine paraffin embedding, sectioned at 5 µm and stained with nuclear fast red or Schmorl's reagent [15].

Cell culture

Osteosarcoma cell lines (SaOS-2, MG63 and TE85) from laboratory stocks were cultured routinely in 9-cm Petri dishes containing DMEM supplemented with 10% fetal calf serum, 50 µg/ml of streptomycin, 50 U/ml of penicillin and 2 mM of L-glutamine (Invitrogen, Paisley, UK). The cells were maintained at 37°C in a humidified atmosphere of 93% air and 7% CO₂.

Identification and quantitation of ochronotic pigment deposition *in vitro*

For experiments in which the deposition of ochronotic pigment was studied, cells were passaged at 4×10^4 cells/well into 24-well plates containing 13-mm diameter sterile coverslips and allowed to grow to confluence for periods up to 28 days with medium change every 3 days. Medium was supplemented with different concentrations of HGA from 0.33 µM to 0.33 mM. At the end of the incubation period, coverslips were washed briefly in isotonic saline solution, fixed in 10% PBFS and stained with Schmorl's stain for 5 min. They were rinsed in distilled water and counterstained in nuclear fast red for 5 min, dehydrated and then mounted using DPX (Sigma-Aldrich). Pigmentation was quantified in two ways. First, individual pigment deposits were counted on digital images of Schmorl's stained cultures and expressed per unit area. Secondly, the area of pigmentation was determined by point counting, using a grid overlain on digital images, and expressed as a proportion of the total area of the cell cultures.

Transmission electron microscopy of HGA-treated cells

MG63 cells were cultured in the absence or presence of 0.33 mM HGA for 21 days. At the end of this period, cells were washed briefly in isotonic saline solution and fixed with 4% paraformaldehyde and 2.5% glutaraldehyde. Cells were then stained with 1% aqueous osmium tetroxide, dehydrated through graded alcohols and embedded into resin (Agar 100, Agar Scientific, Stanstead, UK). Seventy-nanometre sections were cut using a Reichert Ultracut E and post-stained using uranyl acetate and lead citrate. Transmission electron microscopy (TEM) was performed using an FEI 120 kV Tecnai G2 Spirit BioTWIN (FEI Electron Microscopes, Cambridge, UK), and images were obtained using digital image capture with an SIS Megaview III camera (Olympus, Munster, Germany).

Influence of HGA on cell growth and viability

MG63 cells were seeded into 6-well plates at a concentration of 4×10^4 cells per well and grown for 10 days in 2 ml of medium supplemented with different concentrations of HGA from 0.1 µM to 1 mM. Medium was changed after 5 days. At the end of the experimental period, the medium was removed, cleared by centrifugation and stored at -80°C for later detection of P1NP, the amino-terminal pro-peptide of type 1 pro-collagen. Cell layers were washed, trypsinized and counted in disposable haemocytometers (Labtech International, East Sussex, UK). Trypan blue exclusion was used to determine viability.

Measurement of P1NP secretion

P1NP in conditioned medium harvested at the end of the experimental period was assayed using an Elecsys 2010 two-site electro-chemiluminescence immunoassay (Roche, Lewes, UK) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed by analysis of variance using StatsDirect. Differences between groups were determined by Neuman–Keuls post-test.

Results

Schmorl's stain identifies intra- and extracellular ochronotic pigment *in situ*

Ochrotonic pigment deposits were detected as brown granules in the ECM and within fibroblasts in sections of joint capsule from the knee of a patient with AKU (Fig. 1A). Staining with Schmorl's stain, which stained the ochrotonic deposits dark blue–green, enhanced the contrast between pigmented and non-pigmented tissue (Fig. 1B).

Ochrotonic pigment is deposited in cultures of osteosarcoma cells exposed to HGA

When cells from the osteosarcoma lines SaOS-2, MG63 and Te85 were grown in medium containing HGA, there was a dose-dependent darkening of the medium over the 3 days between medium changes, as seen in Fig. 2. In the absence of cells, an equivalent change in colour took place over 3 weeks (data not shown), indicating that cellular activity accelerated pigment formation. Phase-contrast microscopy revealed that cells from all three lines tolerated concentrations of HGA up to 0.33 mM without any evidence of cell toxicity other than slightly reduced growth rate (Fig. 3A–F). Even at 0.33 mM, cells eventually reached confluence, albeit over a longer time period. However, at 1.0 mM there was a major inhibition of cell growth and adhesion (Fig. 3G). In all three cell lines, there was a deposition of ochrotonic pigment in the cells and cell layers with concentrations of HGA from 33 μ M to 0.33 mM. Pigment could be detected as dark deposits in phase contrast (data not shown) and was readily identifiable in fixed tissue following Schmorl's staining, as in Fig. 4B–D. Schmorl's stain did not stain untreated cultures

(Fig. 4A). Quantification of pigment revealed a dose-related increase in pigmentation, either in the number of deposits and/or the total area of pigmentation in the cells and associated matrix from 33 μ M to 0.33 mM HGA (Fig. 5A and B). Pigment was deposited in all three cell lines with the highest deposition in SaOS-2 cells and the lowest in TE85 (data not shown).

TEM reveals intra- and extracellular pigment in HGA-treated cell cultures

TEM examination of MG63 cultures revealed typical cellular structures with an associated network of extracellular fibres. Ochrotonic pigment was detected both intracellularly and extracellularly as numerous small electron-dense deposits. Extracellular pigment deposits were closely associated with the fibrillar structures (Fig. 6).

HGA inhibits cell growth of MG63 cells and viability

There was a dose-dependent inhibition of cell growth up to 0.33 mM HGA, and severe cell toxicity at 1 mM in MG63 cells. Representative results from one experiment are shown in Fig. 7. At HGA concentrations of 0.1 mM, the growth rate of MG63 cells was still 70% of control; there was little evidence of cell toxicity and the cells would reach confluence, albeit slightly slower.

The effect of HGA on the synthesis of collagen

Collagen synthesis, as determined by assay of secreted P1NP and normalized for cell number, increased on treatment with HGA up to 0.33 mM in MG63 cells (Fig. 8). However, there was almost complete inhibition at 1 mM HGA.

Assay of P1NP in a 5-day conditioned culture medium revealed that TE85 secreted the most collagen of the three cell lines. The mean concentration of P1NP in the conditioned medium from confluent 6 wells after 5 days of culture was 150 μ g/l for TE85 cells, compared with 120 μ g/l for MG63 and 20 μ g/l for SaOS-2 cells.

Fig. 1 Schmorl's stain highlights ochrotonic pigment in the joint capsule from the knee of a patient with AKU. **(A)** Section stained with nuclear fast red. Ochrotonic pigment deposits are seen as brown granules in the ECM and within fibroblasts. Examples are indicated by \rightarrow . **(B)** Near serial section with Schmorl's stain. Ochrotonic deposits are stained green. Bar: 50 μ m.

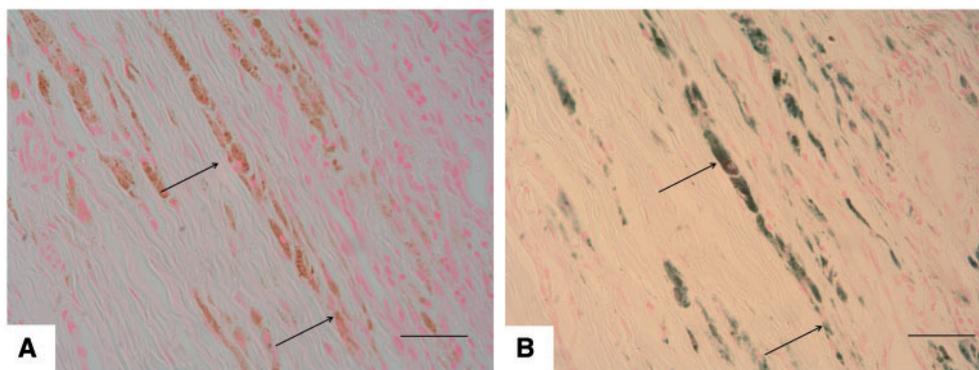


Fig. 2 Macroscopic view of 6-well plates containing MG63 cells cultured for 5 days in different concentrations of HGA. (A) Untreated control, (B) 0.1 μ M HGA, (C) 10 μ M HGA, (D) 33 μ M HGA, (E) 0.1 mM HGA, (F) 0.33 mM HGA and (G) 1 mM HGA. The medium darkened progressively over the 5 days in culture. No change of colouration is seen over the same period in the medium in the absence of cells.

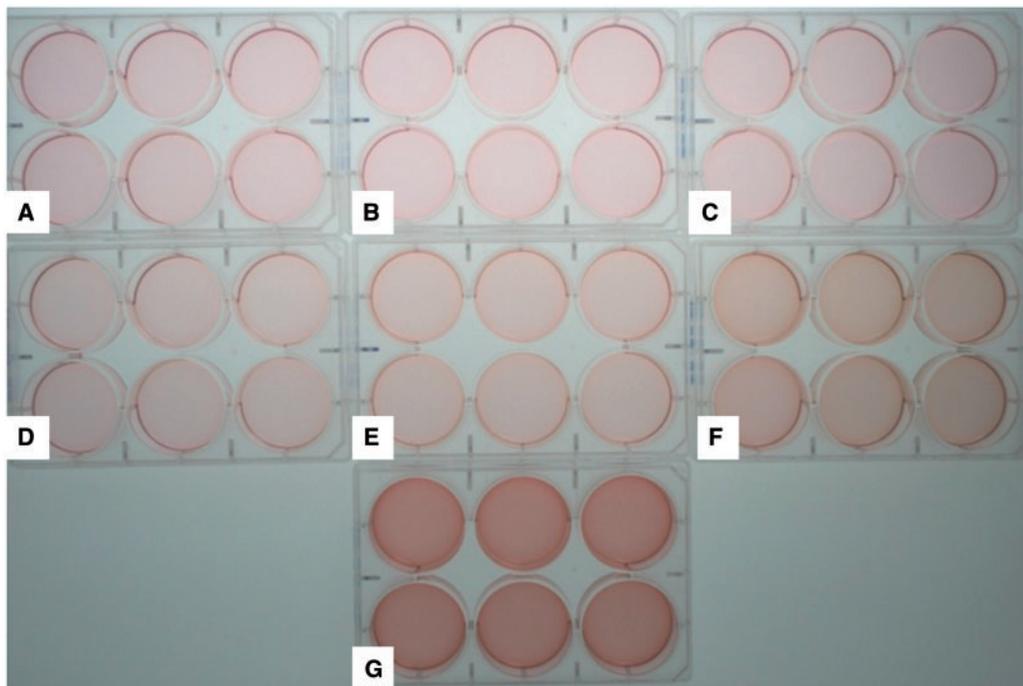
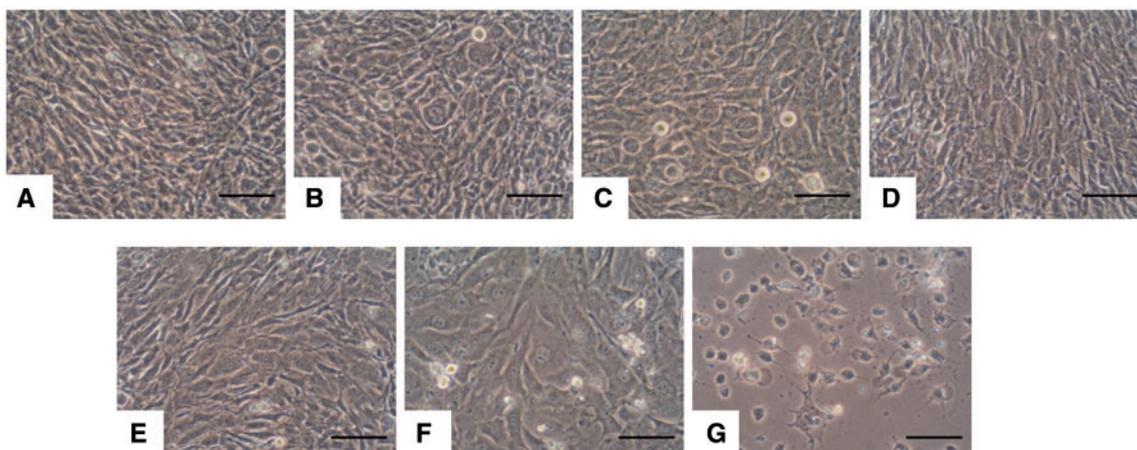


Fig. 3 Phase-contrast photomicrographs of MG63 cells cultured for 5 days treated with different concentrations of HGA. (A) Untreated control, (B) 0.1 μ M HGA, (C) 10 μ M HGA, (D) 33 μ M HGA, (E) 0.1 mM HGA, (F) 0.33 mM HGA and (G) 1 mM HGA. Bar: 50 μ m.

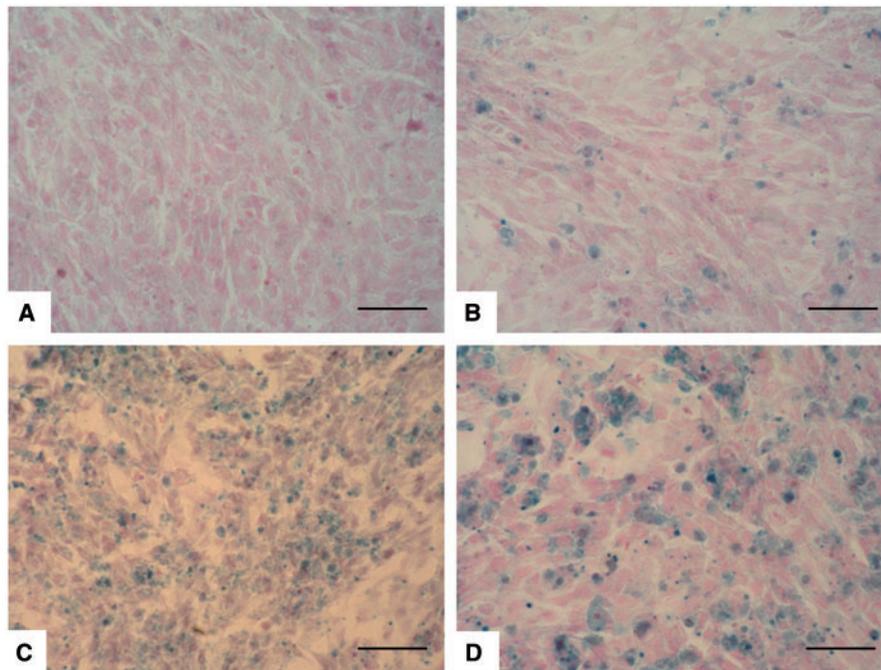


Discussion

The aim of this research was to develop an *in vitro* model of ochronosis. Other researchers have grown connective tissue cells in HGA [16, 17] but there have been no previous reports of pigment deposition *in vitro*. In order to achieve this end, we had to find a technique that would allow the sensitive and reliable identification of ochronotic

pigment in cell cultures. We found that ochronosis in tissue sections from patients with AKU, normally identified as brown granules in the extracellular matrix and within fibroblasts, could be enhanced by Schmorl's stain. This stain produced an intense blue–green colouration that significantly enhanced the contrast between pigmented and non-pigmented tissue. Colour development with Schmorl's stain is dependent on the reduction of

Fig. 4 Schmorl's stain of SaOS-2 cells treated with HGA, showing deposition of intracellular and extracellular deposits of ochronotic pigment 14 days after the addition of HGA. (A) Untreated control, (B) 33 μ M HGA, (C) 0.1 mM HGA and (D) 0.33 mM HGA. Bar: 50 μ m.



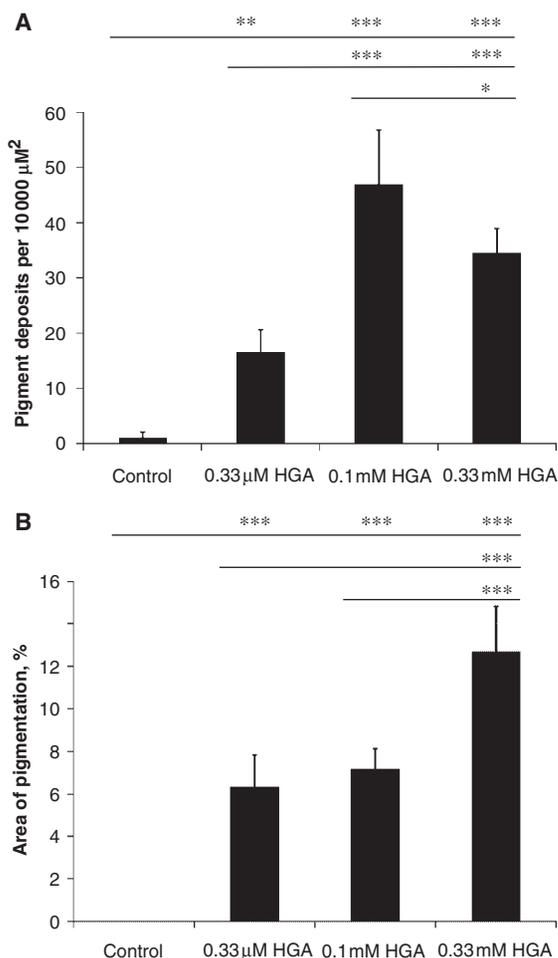
ferricyanide to ferrocyanide, and in tissues is most commonly associated with melanin, emphasizing the structural similarities of ochronotic pigment and melanin. Schmorl's stain was subsequently shown to stain deposits in cultures of osteosarcoma cell lines grown in medium containing HGA. No pigmented deposits were detected in control cultures and the extent of deposition was related to the concentration of HGA indicating that the pigment in the cultures was probably derived from polymerization of HGA. Furthermore, no pigmentation was seen in HGA-supplemented medium not exposed to cells over the same time course, indicating that cellular activity was required for deposition of pigment. The pigment was present intracellularly and extracellularly in the cultures reflecting the observation *in situ*. The relationship between the intra- and extracellular pigmentation is not clear. Although cellular activity appears to be required for pigmentation, the deposition of pigment in the extracellular environment indicated that ECM domains can act as nucleation points for deposition, as previously suggested [17]. Pigment formation in cultures was observed over a range of HGA concentrations from 33 μ M to 0.33 mM, which includes the range observed in AKU patients *in vivo*. However, the time course of deposition *in vitro* of a few days was very rapid compared with pigmentation *in vivo*, which occurs over many years, indicating that there are protective mechanisms in tissues that inhibit pigmentation *in vivo*. In tissues, ECM components are assembled into complex structures in which fibrous proteins, proteoglycans and glycosaminoglycans are associated. This complex association is not replicated

exactly *in vitro* and it is likely that the factors that protect ECM from pigmentation *in vivo* are absent *in vitro* and thus pigmentation is accelerated. Modifying the culture conditions to produce an ECM that is resistant to pigmentation might provide strategies to prevent ochronosis in people with AKU.

Medium containing HGA darkened during the period between medium changes (3–5 days). Again, this was greatly accelerated by cellular activity. Medium with a similar range of concentrations of HGA, incubated at 37°C but not in contact with cells, took several weeks to darken to the same extent.

Pigment was deposited in all three cell lines with the highest deposition in SaOS-2 cells and the lowest in TE85. We used these osteosarcoma cell lines because they are known to secrete ECM components including type I collagen, but the matrix does not mineralize under the culture conditions employed. Within the three cell lines investigated here, pigmentation was greatest in SaOS-2 cell cultures, which have the lowest level of collagen synthesis, indicating that the amount of collagen is not the key factor determining the extent of pigmentation. Unpublished observations have confirmed differential capacity of cell types to induce ochronosis *in vitro*, with even more pigmentation observed in culture of primary chondrocytes and chondrocytic cell lines (Tinti *et al.*, data not published; Taylor *et al.*, data not published). Differences between cell lines reinforce the findings that cellular activity is required for pigment deposition. Furthermore, it might be possible to exploit these differences to identify the mechanism of pigmentation

Fig. 5 Effect of HGA on pigmentation in cultures of SaOS-2 cells. **(A)** Number of pigment depositions per unit area. **(B)** Area of pigment deposition expressed as a per cent of total area. Results are means (s.d.) of six fields. Statistically significant differences between groups are denoted by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.



and to determine therapeutic regimes to prevent ochronosis.

Ultrastructural examination of HGA-treated cultures confirmed the presence of ochronotic-like deposits within cells and associated with a network of extracellular fibres. We have previously observed ochronotic pigment *in situ* in stained, ultrathin sections of tissue as electron-dense shard-like structures found both intracellularly and also connected to collagen fibres in the ECM [18]. The initial deposits in the ECM *in vivo* appear to be associated with the periodicity of the collagen fibres, indicating a nucleation-like process. In contrast, although extracellular fibres could be identified *in vitro*, their morphology was not well ordered and no periodicity was observed. Pigmentation *in vivo* is focal and progressive. Tissues appear to be protected from pigmentation and other factors are required for ochronosis to proceed. In contrast, the disorganized matrix produced *in vitro*

Fig. 6 TEM showing MG63 cells cultured in the presence of 0.33 mM HGA for 19 days. The '→' point to examples of the numerous deposits of ochronotic pigment, which can be identified as electron-dense granules associated with extracellular fibrillar structures and within cells. Bar: 200 nm.

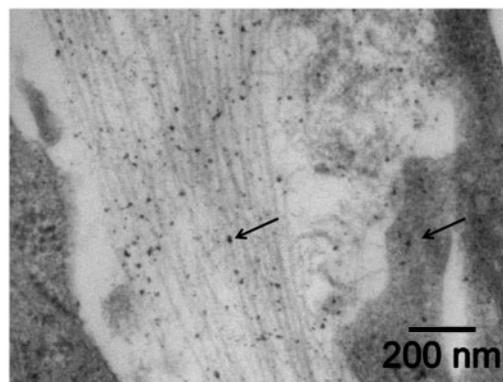
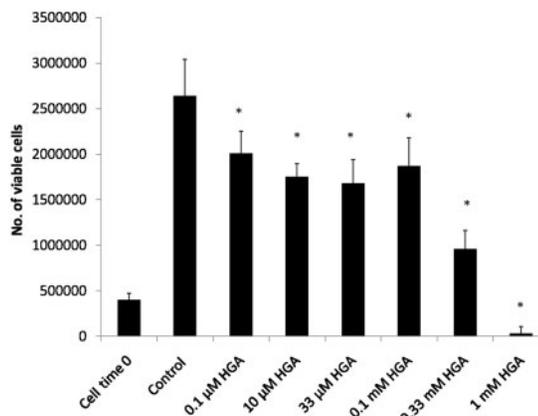


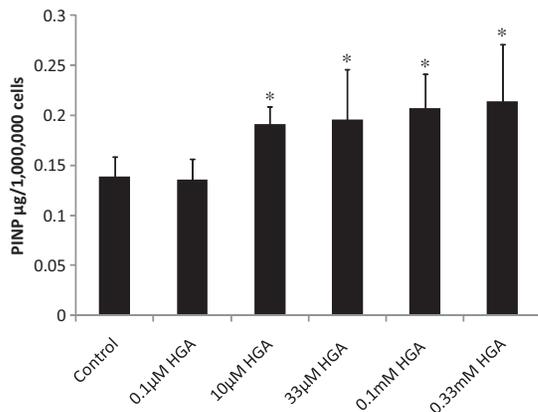
Fig. 7 Effect of HGA on growth and viability of MG63 cells. Cells were seeded into 6-well plates at 40 000 cells per well and grown for 5 days in different concentrations of HGA. Cells were trypsinized, the viability was assessed by Trypan blue exclusion and counted in a haemocytometer. Results are means (s.d.) of six wells. Statistically significant differences from untreated controls are denoted by * $P < 0.01$.



does not appear to be protected and allows pigmentation readily.

Although HGA is clearly toxic at high doses, ochronotic pigment is laid down at concentrations of HGA where there is no evidence of cell toxicity. Furthermore, the presence of HGA in medium up to 0.33 mM increases the synthesis of collagen, when normalized for cell number. Thus, deposition of pigment does not appear to be dependent on cell toxicity or inhibitory effects on the rate of collagen synthesis. In summary, we have developed an *in vitro* model of ochronosis that should contribute to a general understanding of joint destruction in AKU and to the aetiology of OA.

Fig. 8 Effect of HGA on secretion of P1NP release in culture medium of MG63 cell lines treated with different concentrations of HGA. Results are normalized for cell number and are represented as means (s.d.) of six wells. Statistically significant differences from untreated controls are denoted by * $P < 0.01$.



Rheumatology key messages

- An *in vitro* model of ochronosis has been developed.
- Pigmentation *in vitro* is more rapid than *in vivo* indicating that tissues are normally protected from ochronosis.
- Ochronosis requires cellular activity.

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