Experimental Cell Research 342 (2016) 166–174

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Allopurinol and oxypurinol promote osteoblast differentiation and increase bone formation

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ARTICLE INFO

Article history:
Received 13 January 2016
Received in revised form
29 February 2016
Accepted 6 March 2016
Available online 8 March 2016

Keywords:
Bone formation
Allopurinol
Oxypurinol
Osteoblast differentiation

ABSTRACT

Allopurinol and its active metabolite, oxypurinol are widely used in the treatment of gout and hyperuricemia. They inhibit xanthine oxidase (XO) an enzyme in the purine degradation pathway that converts xanthine to uric acid. This investigation examined the effect of allopurinol and oxypurinol on bone formation, cell number and viability, gene expression and enzyme activity in differentiating and mature, bone-forming osteoblasts. Although mRNA expression remained relatively constant, XO activity decreased over time with mature osteoblasts displaying reduced levels of uric acid (20% decrease). Treatment with allopurinol and oxypurinol (0.1–1 μM) reduced XO activity by up to 30%. At these concentrations, allopurinol and oxypurinol increased bone formation by osteoblasts ~4-fold and ~3-fold, respectively. Cell number and viability were unaffected. Both drugs increased tissue non-specific alkaline phosphatase (TNAP) activity up to 65%. Osteocalcin and TNAP mRNA expression was increased, 5-fold and 2-fold, respectively. Expression of NPP1, the enzyme responsible for generating the mineralisation inhibitor, pyrophosphate, was decreased 5-fold. Col1α1 mRNA expression and soluble collagen levels were unchanged. Osteoclast formation and resorptive activity were not affected by treatment with allopurinol or oxypurinol. Our data suggest that inhibition of XO activity promotes osteoblast differentiation, leading to increased bone formation *in vitro*.

1. Introduction

Most of the current treatments for postmenopausal osteoporosis (*e.g.* bisphosphonates, Denosumab) act by inhibiting osteoclast activity and reducing bone resorption, thereby increasing bone mineral density (BMD). In contrast, the only bone anabolic agent currently marketed for treating osteoporosis is the human parathyroid hormone (PTH) analogue teriparatide [1,2]. Since use of PTH is not suitable for all patients [3], additional therapeutic agents which promote bone formation are required.

Allopurinol (1,5-dihydro-4*H*-pyrazole[3,4-*d*]pyrimidin-4-one) and its active metabolite oxypurinol are widely used clinically in the treatment of gout, the most common form of inflammatory arthritis, and hyperuricemia [4,5]. Both agents are purine analogues and act as non-competitive inhibitors of xanthine oxidase (XO), an enzyme in the purine degradation pathway. Febuxostat, which is structurally unrelated to allopurinol, is a non-purine selective XO inhibitor also used to treat gout [6].

Physiologically, XO is involved in many biochemical reactions but its key action is to catalyse the breakdown of hypoxanthine to xanthine and xanthine to uric acid [7]. Inhibition of XO activity reduces the uric acid concentration in the plasma and therefore prevents the development and progression of gout and related conditions [4]. XO expression is widely distributed throughout the body with expression in the liver, gut, lung, kidney, heart and brain [7]. Expression of XO has also been reported in osteoblasts and osteoclasts [8]. Inherited deficiency of XO activity leads to xanthinuria and multiple organ failure characterised by low levels of uric acid and an accumulation of xanthine in tissues [9].

The breakdown of hypoxanthine and xanthine by XO is an oxygen-dependent reaction that also results in the production of the reactive oxygen species (ROS) superoxide (O_2^-) and hydrogen peroxide. XO-derived superoxide can cause oxidative injury to proteins, lipids and DNA, so in preventing its production allopurinol and oxypurinol can act as powerful antioxidants [7]. Previous work seems to suggest that XO activity mainly exerts negative effects on bone. In osteoblast-like cells and bone marrow stromal cells, XO increases oxidative stress leading to reduced cell viability,

DOI of original article: http://dx.doi.org/10.1016/j.jtbi.2015.09.025
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http://dx.doi.org/10.1016/j.yexcr.2016.03.004

an inhibition of differentiation and decreased expression of osteogenic markers [10–12]. Furthermore, osteoblast XO activity is enhanced by inflammatory cytokines including TNFα and IL-1β [8]. XO-derived superoxide has also been shown to stimulate the expression of receptor activator of nuclear factor $_{K}$B ligand (RANKL) in osteoblast-like cells [13]. In osteoclasts, ROS that can be generated by XO have been shown to increase formation and bone resorption [14,15].

Despite gout being a condition that primarily affects the musculoskeletal system, the effects of allopurinol and oxypurinol on bone remain poorly investigated. Whilst there are no studies describing the direct effects of these drugs on bone cell function, allopurinol has been shown to inhibit the increase in bone resorption caused by TNFα and IL-1β [8]. More recently, a combination of allopurinol and another antioxidant, *N*-acetylcysteine, was found to inhibit bone growth in an immobilisation-manipulation model of heterotopic ossification [16].

The aim of this study was to examine the direct effects of allopurinol and oxypurinol on osteoblast and osteoclast survival, differentiation and function, using established *in vitro* methods.

2. Materials and methods

2.1. Reagents

All tissue culture and molecular biology reagents were purchased from Life Technologies (Paisley, UK) unless stated otherwise. Chemical reagents were purchased from Sigma Aldrich (Poole, UK).

2.2. Osteoblast cell culture

Primary rat osteoblast cells were obtained from 2-day-old neonatal Sprague-Dawley rats euthanised by cervical dislocation, as described previously [17,18]. All animal experiments were approved by the University College London Animal Users Committee and the Royal Veterinary College ethics and welfare committee; all animals were maintained in accordance with the UK Home Office guidelines for the care and use of laboratory animals.

Following isolation, cells were resuspended in Dulbecco's Modified Essential Medium, supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin (complete mixture abbreviated to DMEM). Cells were cultured for 2–4 days in a humidified atmosphere of 5% CO_2–95% air at 37 °C in 75 cm^2 flasks until confluent. Upon confluence, cells were sub-cultured into 24-well trays in DMEM supplemented with 2 mM β-glycerophosphate, 50 μg/ml ascorbic acid and 10 nM dexamethasone (supplemented DMEM), with half medium changes every 3 days. Osteoblasts were cultured in the presence of allopurinol and oxypurinol (1 nM–10 μM) to determine the effect on cell proliferation, differentiation, function and gene expression. For the bone formation experiments, cells were also treated with febuxostat and, as a positive control of an anabolic agent, BMP2 (0.1 μM). Unless stated, experiments were carried out at 2 time points during the osteoblast culture; day 7, which represents differentiating osteoblasts, and day 14 (mature, bone forming osteoblasts). All experiments were carefully pH-controlled because bone mineralisation is extremely sensitive to inhibition by acidosis [19]. Bone nodule formation by osteoblasts cultured in 24-well plates was measured by image analysis as described previously [17,18,20].

2.3. Osteoclast cell culture

The long bones were dissected from 6 week-old mice, cut across the epiphyses and the marrow was flushed out with PBS. The resulting suspension was centrifuged at 1500 rpm and resuspended in αMEM supplemented with 100 nM prostaglandin E_2 (PGE_2) and 50 ng/ml macrophage colony stimulating factor (M-CSF). The cell suspension was cultured for 24 h in a 75 cm^2 flask in 5% CO_2/95% atmospheric air to allow attachment of stromal cells and other rapidly adherent cells. The non-adherent cell suspension was removed, centrifuged and resuspended in αMEM supplemented with 100 nM PGE_2, 200 ng/ml M-CSF and 3 ng/ml RANKL (R&D Systems Europe Ltd, Abingdon, UK). Cells were plated onto 5 mm diameter ivory discs (10^6 cells/disc) in 96-multiwells. After 24 h, discs containing adherent osteoclast precursors were transferred to 6 well trays (4 discs/well in 4 ml medium) for a further 6 days at 37 °C in 5% CO_2/95% atmospheric air. Culture medium was acidified to pH ~7.0 by the addition 10 meq/l H^+(as HCL) on day 7 to activate osteoclasts to resorb dentine [21]. Culture medium pH, pCO_2 and PO_2 were monitored throughout using a blood gas analyser (ABL 705, Radiometer, Copenhagen, Denmark). Allopurinol or oxypurinol (1 nM–10 μM) were added for the duration of the culture.

Osteoclasts were fixed in 2% glutaraldehyde and stained to demonstrate tartrate-resistant acid phosphatase (TRAP). Osteoclasts were defined as TRAP-positive cells with 2 or more nuclei and/or clear evidence of resorption pit formation. Osteoclast number and the area resorbed on each disc were assessed 'blind' by transmitted light microscopy and reflective light microscopy and dot-counting morphometry, respectively [21].

2.4. Measurement of xanthine oxidase (XO) activity

Osteoblasts were cultured with 0.1–1 μM allopurinol and oxypurinol for 7 or 14 days. The XO activity of cell lysates was determined colorimetrically using a commercially available kit (XO assay kit, Abcam, Cambridge UK). Total protein in cell lysates was determined using the Bradford assay (Sigma Aldrich, Poole, UK).

2.5. Cell number and viability assay

Osteoblast cell number was measured after 7 and 14 days of treatment with allopurinol and oxypurinol (1 nM–10 μM) using the CytoTox 96® non-radioactive cytotoxicity assay (Promega UK, Southampton UK). This assay quantifies cellular lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released on cell lysis. LDH oxidises lactate into pyruvate, generating NADH, which is then used to convert a tetrazolium salt into a red formazan product in proportion to the number of lysed cells.

Cell supernatants were collected to determine medium LDH levels (cell viability). To establish total cellular LDH levels, cells were lysed with 1% Triton X-100 in water (lysis buffer, 15 μl/ml of medium) for 1 h. The LDH content of the supernatants and cell lysates were measured colorimetrically (490 nm) as per manufacturer's instructions. A standard curve for determination of cell numbers was constructed using cells seeded at 10^2–10^6/well. By expressing medium LDH as a percentage of the total cellular LDH cell viability could be also calculated.

2.6. Measurement of extracellular ATP

Prior to measurement of ATP levels, culture medium was removed, cell layers washed and cells incubated with serum-free DMEM (1 ml/well) for 1 h. Extracellular ATP release was measured luminometrically using the *luciferin-luciferase* assay as previously reported [22]. Cell number and viability were determined as described above.