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Angiopreventive versus angiopromoting effects of allopurinol in the murine sponge model

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ABSTRACT

Recent data has indicated that, besides its classical therapeutic indication in hyperurecemia and gout, xanthine oxidase inhibitors can be used to various forms of ischemia and other types of tissue and vascular injuries. We tested the hypothesis that allopurinol, an inhibitor of xanthine oxidase (XO), might modulate acute and/or chronic inflammatory angiogenesis induced by subcutaneous implantation of synthetic matrix in mice. C57/BL6 male mice (6–7 weeks) were implanted with polyether–polyurethane sponge discs. The animals received by oral gavage 1.0 mg/kg of allopurinol for six consecutive days in two treatment regimen. In the first series of experiments, the treatment was initiated 24 h post-implantation and the implants were removed at day 7 post-implantation. For the assessment of the effect of the compound on chronic inflammation, the treatment was initiated at day 8 post-implantation and the implants removed 14 days post-implantation. Angiogenesis as determined by hemoglobin content, VEGF levels and number of vessels intraimplant, and inflammation (myeloperoxidase —MPO, n-acetyl-β-D-glucosaminidase —NAG, TNF-α and CCL2 levels) were reduced by allopurinol treatment in acute phase. Similarly, the treatment inhibited nitric oxide and H_2O_2 production. However, fibrogenesis determined by collagen deposition and levels of TGF-β1 increased in the implants after allopurinol treatment. In marked contrast with the effects when the treatment initiated 24 h post-implantation, allopurinol increased angiogenesis and inflammation but reduced collagen and TGF-β1 levels intra-implant, when the treatment was started during the chronic inflammatory process. The dual effects of allopurinol described here, extend its range of actions as a potential agent able to modulate the components of the fibrovascular tissue present in both physiological (healing processes) as well as in chronic fibroproliferative diseases. These modulatory effects depended on the phase at which the treatment was initiated.

Introduction

The search for compounds that exert anti-inflammatory, anti-angiogenic, and/or anti-fibrogenic actions has gained clinical importance considering that these are common processes underlying a number of pathological conditions such as chronic inflammation and tumor development. Molecular and biochemical analysis of these pathologies have shown that a number of molecules including, inflammatory enzymes (myeloperoxidase, n-acetyl-B-D-glucosaminidase, xanthine oxidase) and reactive oxygen species (ROS), products of their activities, such as nitric oxide (NO), hydrogen peroxide (H_2O_2), and peroxynitrite, take place in inflammatory events (leukocyte activation/recruitment) and blood vessel formation. Likewise, many cellular products of various cell population within the diseased microenvironment including cytokines (TNF-α, VEGF, TGF-β1) are essential for excessive angiogenesis and extracellular matrix deposition in pathological conditions (Aldaba-Muruato et al., 2013; Gormus et al., 2013; Kou et al., 2008). Thus, these events inflammation, angiogenesis, and fibrogenesis are the potential targets to address disease process where they co-exist and are contributory to disease progression. Exploration of inhibition strategies of these events is expected to contribute to therapeutic interventions in a number of pathological processes.

A wide range of anti-angiogenic or anti-inflammatory compounds has been described and many of them were primary designed for totally different pharmacological purposes. This drug repositioning/repurposing concept, i.e., finding new indication for existing drugs is of commercial and medical value as it presents an excellent strategy to achieve optimal potential and maximize the value of a therapeutic drug (Ashburn and Thor, 2004).

One such drug, the xanthine oxidase (XO) inhibitor allopurinol (1,5-dihydro-4H-pyrazolo [3,4-d]pyrimidin-4-one) has been the cornerstone of the clinical management of gout and conditions associated with hyperuricemia for several decades (Khanna and FitzGerald, 2015; Klinenberg et al., 1965; Pacher et al., 2006). However, more recent data have indicated the use of xanthine oxidase inhibitors to various

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forms of ischemia and other types of tissue and vascular injuries, inflammatory diseases, chronic heart failure (Berry and Hare, 2004; Harrison, 2002; Harrison, 2004). Allopurinol and its active metabolite oxypurinol has shown considerable promise in the treatment of these conditions both in experimental animals and in small-scale human clinical trials (Aldaba-Muruato et al., 2013; Khanna and FitzGerald, 2015; Kim et al., 2015; Margaritis et al., 2011; Pacher et al., 2006; Peto et al., 2005). The main mechanisms by which allopurinol exerts its beneficial effects involve decreased generation of superoxide, hydrogen peroxide and uric acid. In addition, there is evidence that XO can catalyze the reduction of nitrite and nitrate (normally the decomposition products of NO) back to NO (Godber et al., 2000; Li et al., 2005; Millar et al., 1998, 2002; Zhang et al., 1998), an important mechanism involved in vascular homeostasis.

Collectively, these reports have expanded the range of actions of allopurinol beyond its acid uric lowering effect to pathological conditions involving vascular and inflammatory processes. In addition, some evidence has indicated that XO inhibitors modulate angiogenesis negatively, for example, reducing endothelial viability, proliferation, and vascular tube formation (Kou et al., 2008).

Thus, based on the reported actions of allopurinol modulating either positively or negatively proliferation and migration of a number of cells involved in fibroproliferative pathological processes, we reasoned that it might also modulate inflammatory angiogenesis in the mouse sponge model. In this model, the acellular and avascular synthetic matrix implanted subcutaneously in the dorsal of the animals induces the migration, proliferation and activation of various cells types responsible for the development of a fibrovascular tissue that underlies both acute and chronic pathological conditions (Almeida et al., 2014; Castro et al., 2012, 2014; Marques et al., 2011). We report here that allopurinol exhibited both angiopreventive and angiopromoting effects on the fibrovascular tissue induced by synthetic matrix in mice. The contrasting findings were dependent on the phase in which the treatment was initiated.

Material and methods

Animals

Male C57/BL6 mice 7–8 weeks of age (20–25 g body weight), provided by the Centro de Bioterismo (CEBIO) of Universidade Federal de Minas Gerais (UFMG)-Brazil, were used in these experiments. The animals were housed individually and provided with chow pellets and water ad libitum. The light/dark cycle was 12:12 h with lights on at 7:00 a.m. and lights off at 7:00 pm. Efforts were made to avoid all unnecessary distress to the animals. Housing, anesthesia, and postoperative care complied with the guidelines established by our local Institutional Animal Welfare Committee (process number: CEUA n° 007/11).

Sponge discs implantation and Allopurinol treatment

Polyether–polyurethane sponge (Vitafoam Ltd., Manchester, UK) was used as the implanted material. The implants were in the shape of discs, 5 mm thick x 8 mm diameter. They were soaked overnight in 70% v/v ethanol and then sterilized by boiling in distilled water for 15 min before implantation. The animals were anesthetized with a mixture of ketamine 150 mg/kg and xylazine 10 mg/kg and the dorsal hair shaved and the exposed skin wiped with 70% ethanol. The sponge discs were aseptically implanted into a subcutaneous pouch, which had been made with curved artery forceps through a 1-cm long dorsal mid-line incision. Post-operatively, the animals were monitored for any signs of infection at the surgical site, discomfort, or distress; any animals showing such signs were promptly euthanized. Allopurinol 1 mg/Kg body weight was administered by oral gavage daily for 6 days in two-treatment regimen. The dose of the compound was chosen based on pilot experiments and on the range used in humans for treatment of gout (Bieber and Terkeltaub, 2004). In the first series of the experiments, in twenty five animals, the treatment was initiated 24 h post-implantation and the implants were removed at day 7 post-implantation. For the assessment of the effect of the compound on chronic inflammation, in twenty mice, the treatment was initiated at day 8 post-implantation and the implants removed 14 days post-implantation. After removal, the implants were weighed and processed to evaluate vascularization (hemoglobin content, VEGF levels and number of vessels), inflammatory markers (Nitric oxide production, MPO and NAG activities, and chemokines levels), and fibrogenesis markers (total collagen soluble and TGF-β1 levels).

Hemoglobin extraction (Hb)

The extent of vascularization of the sponge implants was assessed by the amount of hemoglobin (Hb) detected in the tissue using the Drabkin method (Araujo et al., 2011; Marques et al., 2011). After careful removal of the sponge implants, they were cleared of any adherent tissue and weighed. Each implant was homogenized (Tekmar TR-10, OH) in 1 ml of Drabkin reagent (Labtest, São Paulo, Brazil) and centrifuged at 12,000 ×*g* for 20 min. The supernatants were filtered through a 0.22-μm Millipore filter. The hemoglobin concentration in the samples was determined spectrophotometrically by measuring absorbance at 540 nm using an ELISA plate reader and comparing it against a standard hemoglobin curve. Hemoglobin content in the implant was expressed as μg Hb per mg wet tissue.

Measurement of VEGF, TNF-α, and TGF-β1 production in the sponge implants

The implants were homogenized in PBS pH 7.4 containing 0.05% Tween, and centrifuged at 10,000 ×*g* for 30 min. The levels of the cytokines in the supernatant from each implant were measured in 50 μl of the supernatant using Immunoassay Kits (R and D Systems, USA) and following the manufacturer's protocol. Briefly, dilutions of cell-free supernatants were added in duplicate to ELISA plates coated with a specific murine monoclonal antibody against cytokine, followed by the addition of a second horseradish peroxidase-conjugated polyclonal antibody, also against cytokine. After washing to remove any unbound antibody-enzyme reagent, a substrate solution (50 μL of a 1:1 solution of hydrogen peroxide and tetramethylbenzidine 10 mg/ml in DMSO) was added to the wells. Color development was halted after 20 min incubation with 2 N sulfuric acid (50 μL) and the intensity of the color was measured at 540 nm on a spectrophotometer (E max — Molecular Devices). Standards were 0.5-$\log_{10}$ dilutions of recombinant murine cytokines from 7.5 pg ml^{-1} to 1000 pg ml^{-1} (100 μl). The threshold of sensitivity for each chemokine is 15.625 pg/mL. The results were expressed as pg cytokine per mg wet tissue.

Tissue extraction and determination of myeloperoxidase and N-acetyl-β-D-glucosaminidase activities

The content of neutrophils in implants was measured by assaying myeloperoxidase (MPO) activity as previously described (Araujo et al., 2010). The implants were weighed, homogenized in pH 4.7 buffer (0.1 M NaCl, 0.02 M $NaPO_4$, 0.015 M NaEDTA), and centrifuged at 12,000 ×*g* for 10 min. The pellets were then re-suspended in 0.05 M $NaPO_4$ buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) followed by three freeze–thaw cycles using liquid nitrogen. MPO activity in the supernatant samples was assayed by measuring the change in absorbance (optical density; OD) at 450 nm using tetramethylbenzidine (1.6 mM) and H_2O_2 (0.3 mM). The reaction was terminated by adding 50 μl of H_2SO_4 (4 M). Results were expressed as a change in OD/g wet tissue.

Infiltration of mononuclear cells into the implants was quantified by measuring the levels of the lysosomal enzyme N-acetyl-β-D-