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Acetyl-11-keto-β-boswellic acid reduces retinal angiogenesis in a mouse model of oxygen-induced retinopathy

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ABSTRACT

Retinal diseases characterized by pathologic retinal angiogenesis are the leading causes of blindness worldwide. Although therapies directed toward vascular endothelial growth factor (VEGF) represent a significant step forward in the treatment of proliferative retinopathies, further improvements are needed. In the last few years, an intense research activity has focused around the use of herbal and traditional natural medicines as an alternative for slowing down the progression of proliferative retinopathies. In the present study, we investigated the antiangiogenic effects of acetyl-11-keto-β-boswellic acid (AKBA), one of the active principles derived from the plant *Boswellia serrata*, used in Ayurvedic systems of medicine. We studied the antiangiogenic properties of AKBA using the mouse model of oxygen-induced retinopathy (OIR), which mimics the neovascular response seen in human retinopathy of prematurity. We first evaluated the effects of subcutaneously administered AKBA on the expression/activity of proteins which are known to play a role in the OIR model. In the retina, AKBA increased expression and activity of Src homology region 2 domain-containing phosphatase 1 and reduced the phosphorylation of the transcription factor signal transducer and activator of transcription 3 (STAT3) as well as VEGF expression and VEGF receptor (VEGFR)-2 phosphorylation. Likely as a result of these effects, AKBA significantly reduced retinal neovascularization in OIR mice without affecting retinal cell survival and retinal function. Using retinal explants cultured in hypoxia and an activator of STAT3 phosphorylation, we showed that the AKBA-induced inhibition of VEGFR-2 phosphorylation is likely to be mediated by a mechanism depending on an SHP-1/STAT3/VEGF axis. In the OIR model, neovascularization results from the activation of retinal endothelial cells, therefore we evaluated whether AKBA affected the angiogenic response of human retinal microvascular endothelial cells (HRMECs). We observed that AKBA reduced proliferation, migration and tube formation in HRMECs stimulated with exogenous VEGF, while it reduced migration and tube formation in untreated HRMECs. Taken together, our results demonstrate the antiangiogenic effects of AKBA in a model of pathologic neovascularization, providing a rationale for further investigation of AKBA as a promising therapeutic agent to reduce the impact of proliferative retinopathies.

Abbreviations: AKBA, acetyl-11-keto-β-boswellic acid; DMEM, Dulbecco's modified eagle's medium; DMSO, dimethyl sulfoxide; EBM-2, Endothelial Basal Medium-2; ELISA, enzyme-linked immunosorbent assay; ERG, electroretinogram; FBS, fetal bovine serum; HRMECs, human retinal microvascular endothelial cells; OD, optical density; OIR, oxygen-induced retinopathy; OPs, oscillatory potentials; OSM, oncostatin M; PB, phosphate buffer; PBS, phosphate buffered saline; PND, postnatal day; PVDF, polyvinylidene difluoride; QPCR, quantitative real-time RT-PCR; SHP-1, Src homology region 2 domain-containing phosphatase 1; STAT3, signal transducer and activator of transcription 3; pSTAT3, phosphorylated form of STAT3; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor 2.

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1. Introduction

Pathological retinal angiogenesis is a major cause of vision loss in various diseases, including retinopathy of prematurity, diabetic retinopathy, and age-related macular degeneration (Gariano and Gardner, 2005). Vascular endothelial growth factor (VEGF) plays major roles in neovascular eye diseases, prevalently acting at VEGF receptor (VEGFR)-2 (Witmer et al., 2003). In these diseases, stress conditions caused by ischemia, excitotoxicity and reactive oxygen species induce VEGF release principally by Müller cells (Yanni et al., 2010; Bai et al., 2009; Yafai et al., 2004), astrocytes (Weidemann et al., 2010) or by retinal neurons (Cervia et al., 2012). The released VEGF then would act at VEGFR-2 expressed by endothelial cells to induce a neovascular response. Thus, VEGF has become an ideal target for therapies used to counteract retinal angiogenesis (Miller et al., 2013). However, anti-VEGF agents, administered through intravitreal injections, can induce several serious ocular side effects including retinal fibrosis, inflammation and endophtalmitis and may interfere with neuronal survival in the retina since VEGF is a recognized factor with neuroprotective actions (reviewed in Casini et al., 2014). An additional concern is the potential for upregulation of VEGF receptors or compensatory overproduction of VEGF that may follow the prolonged use of anti-VEGF therapy, potentially resulting in a rebound effect at the discontinuation of treatment (Agustin et al., 2007). Therefore, the exploration and evaluation of new antineovascularization compounds is greatly needed.

In the last few years, an intense research activity has focused around the use of herbal and traditional natural medicines as an alternative for slowing down the progression of proliferative retinopathies (Song et al., 2012). Acetyl-11-keto-β-boswellic acid (AKBA) is one of the active principles present in boswellic acids derived from the plant *Boswellia serrata*, which have been used in Ayurvedic systems of medicine against a number of inflammatory diseases. Recently, it has been reported that AKBA inhibits tumor growth and production of angiogenic markers in mice (Yadav et al., 2012a; Park et al., 2011a,b; Pang et al., 2009a) with an anti-cancer effect that includes inhibition of VEGFR-2 signaling (Pang et al., 2009a) and may be due, at least in part, to the inhibition of intracellular signaling pathways involving signal transducer and activator of transcription 3 (STAT3). This effect is mediated by the induction of the Src homology region 2 domain-containing phosphatase 1 (SHP-1), which dephosphorylates STAT3 (Kunnumakkara et al., 2009). STAT3 is a cytoplasmic protein that, once phosphorylated in response to different stimuli, including hypoxia, forms a dimer and translocates to the nucleus, where it activates the transcription of several target genes, including VEGF (Chen and Han, 2008). The inhibition of STAT3 activation by AKBA has been shown to lead to the suppression of several gene products, including VEGF, in human multiple myeloma cell lines (Kunnumakkara et al., 2009). Interestingly, we have recently observed that AKBA increases the expression of SHP-1 and reduces both VEGF content and release in mouse retinal explants (Mei et al., 2012). In addition, we have also demonstrated that activation of SHP-1 as well as reduction of STAT3 phosphorylation are causally linked to the effects of octreotide, an analogue of the peptide somatostatin, which reduces retinal neovascularization in the mouse model of oxygen-induced retinopathy (OIR), a model that mimics the neovascular response seen in retinopathy of prematurity (Dal Monte et al., 2010; Mei et al., 2012). Together, these observations suggest that AKBA may be effective in counteracting the development of new blood vessels in OIR retinas.

In the present work, we performed a series of studies in *in vivo* and *ex vivo* retinal models to evaluate the effects of AKBA on SHP-1 expression/activity, STAT3 phosphorylation, VEGF expression, and VEGFR-2 activation to test the hypothesis that AKBA may play an anti-angiogenic effect acting on an SHP-1/STAT3/VEGF axis. In addition, to test the hypothesis that AKBA may exert some of its effects directly on endothelial cells, in agreement with previous findings (Yadav et al., 2012a; Park et al., 2011a,b; Pang et al., 2009a), we evaluated the effects of AKBA on proliferation, migration and tube formation of human retinal microvascular endothelial cells (HRMECs).

2. Materials and methods

2.1. Materials

The Endothelial Basal Medium-2 (EBM-2) and endothelial growth factors (Microvascular Endothelial Cell Growth Medium-2, EGM-2MV SingleQuot, without addition of gentamicin) as well as the Dulbecco's modified eagle's medium (DMEM) were from Lonza (Allendale, NJ). The fetal bovine serum (FBS), the mouse monoclonal antibody directed to β-actin, the rabbit anti-mouse horseradish peroxidase-labeled secondary antibody, oncostatin M (OSM) and all the other chemicals, unless otherwise specified, were purchased from Sigma–Aldrich (St. Louis, MO). The Millicell-CM culture inserts and the enhanced chemiluminescence reagent were from Millipore (Billerica, MA). AKBA was from PhytoPlan (Heidelberg, Germany). The RNeasy Mini Kit, the QuantiTect Reverse Transcription Kit and the SYBR Green PCR Kit were from Qiagen (Valencia, CA). The protease inhibitor cocktail Complete, the phosphatase inhibitor cocktail PhosStop and the cell proliferation reagent WST-1 were from Roche Applied Science (Indianapolis, IN). Polyvinylidene difluoride (PVDF) membrane was obtained from Bio-Rad Laboratories (Hercules, CA). The mouse monoclonal antibodies directed to SHP-1 (cat # 610125) and cytochrome c (cat # 556433), and the Matrigel were from BD Biosciences (San Diego, CA). The mouse monoclonal antibodies directed to the phosphorylated form of STAT3 (pSTAT3; cat # sc-8059) and of VEGFR-2 (pVEGFR-2; cat # sc-16628), the rabbit polyclonal antibodies directed to STAT3 (cat # sc-482), VEGF (cat # sc-7015) and VEGFR-2 (cat # sc-504), and the mouse anti-rabbit horseradish peroxidase-labeled secondary antibody were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). The enzyme-linked immunosorbent assay (ELISA) kit for the detection of VEGF was obtained from R&D Systems (Minneapolis, MN). The rat monoclonal antibody directed to CD31 was obtained from BD Pharmingen (San Diego, CA; cat # 550274). The secondary antibody Alexa Fluor 488 was from Molecular Probes (Eugene, OR).

2.2. Animals

The procedures involving animals were approved by the Ethics Committee in Animal Experiments of the University of Pisa and were carried out in agreement with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in compliance with the Italian guidelines for animal care (DL 116/92) and the EU Directive (2010/63/EU). All efforts were made to reduce both animal suffering and the number of animals used. Two month-old male and female mice (C57BL/6J strain) were originally purchased from Charles River Laboratories Italy (Calco, Italy) and were mated in our breeding colony. Experiments were performed on a total of 81 mouse pups of both sexes. Animals were kept in a regulated environment (23 ± 1 °C, 50 ± 5% humidity) with a 12-h light/dark cycle (lights on at 8 AM) with food and water ad lib. In all experiments, mice were anesthetized with halothane (4%), sacrificed by cervical dislocation and the eyes were enucleated.