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Inhibitory effects of rosmarinic acid on pterygium epithelial cells through redox imbalance and induction of extrinsic and intrinsic apoptosis

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ABSTRACT

Pterygium is a common tumor-like ocular disease, which may be related to exposure to chronic ultraviolet (UV) radiation. Although the standard treatment for pterygium is surgical intervention, the recurrence rate of pterygium is high when no effective inhibitory drug is used after surgery. Rosmarinic acid (RA) is a polyphenol antioxidant with many biological activities, including anti-UV and anti-tumor properties. This study aimed to examine the inhibitory effects of RA on pterygium epithelial cells (PECs). Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was used to examine the cell cytotoxicity of PECs after RA treatment. A fluorescent probe, DCFH-DA (2′,7′-dichlorofluorescin diacetate), was stained with PECs to measure intracellular reactive oxygen species (ROS) levels. Antioxidant activity assays were used to measure the levels of superoxide dismutase (SOD) and catalase (CAT) in PECs. Western blot analysis was used to determine the protein expression of nuclear factor E2-related factor 2 (Nrf2), heme oxygenase 1 (HO-1), quinone acceptor oxidoreductase 1 (NQO1), and apoptosis-associated proteins. RA significantly reduced the cell viability of the PECs. Treatment with RA remarkably increased the Nrf2 protein expression levels in the nucleus, HO-1 and NQO1 protein expression levels, and the activities of SOD and CAT. As a result, intracellular ROS levels in PECs were decreased. Additionally, the induction of extrinsic apoptosis on PECs by RA was associated with increasing expressions levels of Fas, Fas-associated protein with death domain (FADD), tumor necrosis factor-alpha (TNF-α), and caspase 8 protein. Moreover, the induction of intrinsic apoptotic cell death in PECs was confirmed through upregulation of cytochrome *c*, Bax, caspase 9, and caspase 3 and downregulation of Bcl-2 and pro-caspase 3. Our study demonstrated that RA could inhibit the viability of PECs through regulation of extrinsic and intrinsic apoptosis pathways. Therefore, RA may have potential as a therapeutic medication for pterygium.

1. Introduction

Pterygium, a wing-shaped lesion that originates from the conjunctiva (Coroneo et al., 1999), causes severe visual impairment through its excessive central migration into the visual axis (Gazzard et al., 2002). Currently, the main treatment for pterygium is surgical excision (Ang et al., 2007); pterygium has a recurrence rate of 40–80% and is more aggressive than the primary pterygium (Kareem et al., 2012). Pterygium shows tumor-like characteristics (Tung et al., 2010), including high cell proliferation in its epithelium (Kase et al., 2007) and overexpression of anti-apoptosis proteins, including p53, survivin, and Bcl-2, which causes the growth and development of pterygium (Tan et al., 2000; Zhang et al., 2011b).

Although the pathogenesis of pterygium is still being investigated, many studies have reported that the development of pterygium is related to chronic ultraviolet (UV) radiation exposure (Bradley et al., 2010). UV radiation induces cellular reactive oxygen species (ROS) generation. Overproduction of ROS leads to oxidative stress, which can injure cellular DNA, lipids, and proteins (Bergendi

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et al., 1999; Kumar et al., 2012). Compared to the normal conjunctiva, pterygium has a higher expression of oxidative stress markers (Sano et al., 2013; Tsai et al., 2005) and lower antioxidant enzyme activity (Balci et al., 2011). Therefore, the imbalance of the oxidation-antioxidant system is associated with the formation of pterygium (Sacca et al., 2013).

Many studies have reported that natural products are efficacious in the prevention of oxidative stress-related pathologies due to particular bioactivity effects. A major defense mechanism for the prevention and treatment of oxidative stress-related pathologies comprises reducing the production of reactive metabolites by raising the levels of the endogenous antioxidant defense system. Because there is no effective medication to inhibit pterygium formation and the pathogenesis of pterygium is related to oxidative stress, it is necessary to discover novel antioxidants for pterygium.

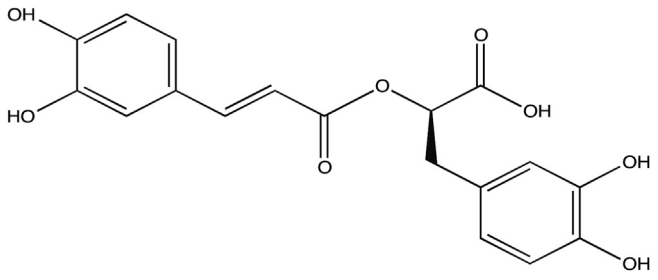

Fig. 1. Structure of rosmarinic acid.

Rosmarinic acid (RA) is an ester of caffeic acid and 3,4-dihydroxy-phenyllactic acid (Fig. 1) (Petersen and Simmonds, 2003), which has been found in more than 240 plant species (Petersen et al., 2009). RA has several biological activities, including anti-inflammatory, anti-viral, anti-bacterial, anti-tumoral, and anti-angiogenesis properties. Many reports have indicated that RA serves as a photo-protective agent against UV exposure due to its inhibitory effects against skin photocarcinogenesis *in vivo* (Sanchez-Campillo et al., 2009) and the prevention for UVB-induced DNA damage *in vitro* (Vostalova et al., 2010). Moreover, RA can inhibit cell proliferation and induce cell apoptosis of hepatic stellate cells (Zhang et al., 2011a), and it also has the ability to induce lymphoblastic leukemia cell death through a different cell death pathway (Wu et al., 2015).

On the basis of the biological activities of RA found both *in vitro* and *in vivo*, the aim of the present study was to evaluate the inhibitory effects of RA in pterygium epithelial cells (PECs) and to elucidate the molecular mechanisms of RA in enhancing the anti-oxidant defense system and inducing cell apoptosis pathways in PECs.

2. Materials and methods

2.1. Reagents

RA was obtained from Sigma-Aldrich. Anti-β-actin was purchased from GeneTex, and anti-pan-Cytokeratin Alexa Fluor® 488,

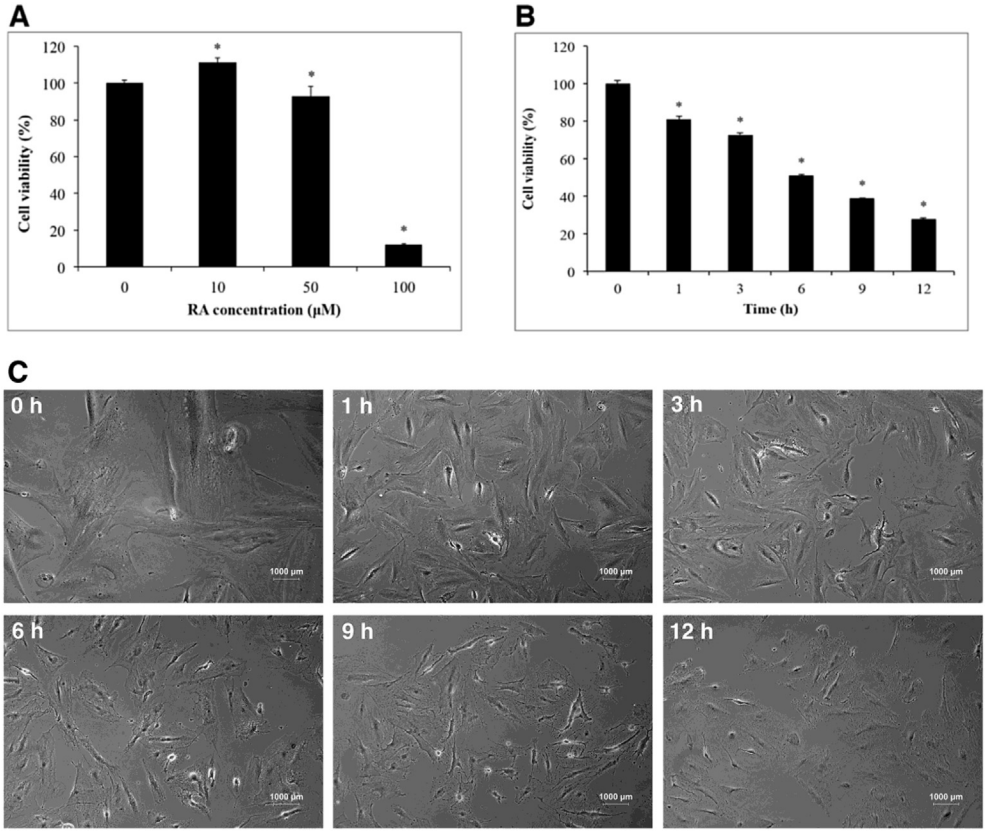

Fig. 2. RA inhibits cell viability in pterygium epithelial cells (PECs). (A) PECs were plated in 24-well culture plate at 80% confluence and treated with 10, 50 and 100 μM RA for 24 h. Cell viability was assessed by MTT assay in different concentrations of RA-treated PECs. (B) PECs were plated in 24-well culture plate at 80% confluence and treated with 100 μM RA for 0 h, 1 h, 3 h, 6 h, 9 h, 12 h. Cell viability was determined using MTT assay in RA-treated PECs for different time periods. The cell viability (%) was calculated as: (A_{540} of treated cells/A_{540} of untreated cells) x 100. (C) Cell morphology of PECs was evaluated with an inverted microscopy after RA treatment at different time periods. * Significantly different from 0 μM (untreated) group ($p < 0.01$).