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Therapeutic effects of zerumbone in an alkali-burned corneal wound healing model

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ABSTRACT

Cornea is an avascular transparent tissue. Ocular trauma caused by a corneal alkali burn induces corneal neovascularization (CNV), inflammation, and fibrosis, leading to vision loss. The purpose of this study was to examine the effects of Zerumbone (ZER) on corneal wound healing caused by alkali burns in mice. CNV was induced by alkali-burn injury in BALB/C female mice. Topical ZER (three times per day, 3 μl each time, at concentrations of 5, 15, and 30 μM) was applied to treat alkali-burned mouse corneas for 14 consecutive days. Histopathologically, ZER treatment suppressed alkali burn-induced CNV and decreased corneal epithelial defects induced by alkali burns. Corneal tissue treated with ZER showed reduced mRNA levels of pro-angiogenic genes, including vascular endothelial growth factor, matrix metalloproteinase-2 and 9, and pro-fibrotic factors such as alpha smooth muscle actin and transforming growth factor-1 and 2. Immunohistochemical analysis demonstrated that the infiltration of F4/80 and/or CCR2 positive cells was significantly decreased in ZER-treated corneas. ZER markedly inhibited the mRNA and protein levels of monocyte chemoattractant protein-1 (MCP-1) in human corneal fibroblasts and murine peritoneal macrophages. Immunoblot analysis revealed that ZER decreased the activation of signal transducer and activator of transcription 3 (STAT3), with consequent reduction of MCP-1 production by these cells. In conclusion, topical administration of ZER accelerated corneal wound healing by inhibition of STAT3 and MCP-1 production.

1. Introduction

The cornea is an avascular transparent tissue, a characteristic referred to as corneal “angiogenic privilege;” this avascularity promotes visual acuity. Ocular trauma resulting from a corneal alkali burn causes corneal epithelial cell defects, scar formation, angiogenesis, tissue inflammation, and fibrosis, leading to reduced corneal transparency and vision loss [1]. The balance between proangiogenic and antiangiogenic molecules is essential for corneal avascularity and optical clarity [2,3]. Because pathologic angiogenesis is an important event in the proliferative phase of corneal wound healing, the imbalance of these factors caused by a chemical burn triggers the onset of corneal neovascularization (CNV).

The infiltration of large numbers of neutrophils and macrophages is closely related to the development of neovascularization. However, a recent study demonstrated that alkali-induced CNV developed independently of granulocyte infiltration [4]. The recruitment of monocyte/macrophages, which are presumed to be a major source of angiogenic molecules, is regulated by the coordinated action of chemokines and adhesion molecules [5]. Mouse monocytes/macrophages express a distinct set of chemokine receptors, including CCR2, CCR5 and CX_3CR1, on their cell surfaces [6]. Several studies have suggested that $CCR2^{-/-}$ and $CCR5^{-/-}$ mice exhibit reduced CNV, indicating that these chemokine receptor-positive cells could contribute to angiogenesis [7,8]. On the contrary, $CX_3CR1^{-/-}$ mice exhibit enhanced CNV despite reduced macrophage infiltration [6].

Among the chemokines associated with these receptors, monocyte chemotactic protein-1 (MCP-1)/CCL2, which is a ligand for CCR2 and is expressed on inflamed endothelial cells, epithelial cells, macrophages, and stromal keratocytes, plays a critical role in corneal inflammation-related diseases [9,10]. This chemokine can induce the migration of monocytes/macrophages, neutrophils, and T cells to sites of inflammation via activation of CCR2. Accumulating evidence supports a role for signal transducer and activator of transcription 3 (STAT3) in the expression of MCP-1 through binding to and activating its promotor [11]. Furthermore, corneal sterile inflammation-induced activation of the IL-6 trans-signaling pathway induces phosphorylation of STAT3 and consequently increases the production of MCP-1 in corneal fibroblasts

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[12]. Therefore, the inhibition of this signaling molecule may have beneficial effects on corneal inflammation and wound healing after corneal injury.

Zerumbone (ZER) is a cyclic sesquiterpene that is a key component of the rhizomes of wild ginger, *Zingiber zerumbet* Smith [13]. It has been reported that ZER has many biological and pharmacological properties. Recent studies revealed that zerumbone exerted antitumor activities, including inhibition of cancer cell proliferation [14], suppression of tumor invasion, and induction of cancer cell apoptosis [15,16]. Also, this compound exerts anti-inflammatory [17–19] and anti-oxidant effects [18,20]. Dietary ZER prevents ultraviolet B (UVB)-induced corneal damage by inhibition of nuclear factor-κB (NF- κB), inducible nitric oxide synthase, and tumor necrosis factor-α, with a concomitant reduction of malondialdehyde accumulation and oxidative stress in a mouse model [18]. Despite these potential beneficial effects, local administration of ZER to inflammation-related angiogenic reactions on the ocular surface and its effects on chemokine-related macrophage infiltration have not been investigated. Therefore, the purpose of this study was to investigate the effects of ZER on corneal wound healing caused by alkali burns in mice.

2. Materials and methods

2.1. Animals

Eight-week-old female BALB/c mice were purchased from Samtako (South Korea). Mice were maintained in standard conditions (24 ± 2 °C, 50 ± 5% humidity) in a pathogen-free environment with a 12-hour light/dark cycle. Experimental and animal management procedures were approved by the Institutional Animal Care and Use Committee of Chonbuk National University. The animal facility of Chonbuk National University is fully accredited by the National Association of Laboratory Animal Care.

2.2. Alkali-induced corneal injury model

Mice were anesthetized with an intramuscular injection of Zoletil at a dose of 0.2 ml/kg body weight. A piece of Whatman #3 filter paper (2 mm diameter) soaked in 1 N NaOH was placed in the center of the cornea of the left eye for 40 s. The ocular surface was then rinsed with 25 ml of PBS. After the alkali burn injury, the animals were randomly divided into four groups of equal size. Mice in one group were topically administered 0.1% DMSO (three times per day, 3 μl each time), and the others were topically administered ZER (Sigma-Aldrich, ST. Louis, MO, USA) using a pipette (three times per day, 3 μl each time, at concentrations of 5, 15, and 30 μM). At the indicated time intervals (2, 4, and 7 days), mice were sacrificed and their corneas were removed. The corneas were stored at −80 °C and used for RNA extraction. In another series of experiments, mice were sacrificed at the indicated time points (4, 7, and 14 days) after alkali treatment and the eyes were entirely removed from each animal. These eyes were fixed in 10% phosphate-buffered formalin for histological analysis.

2.3. Biomicroscopic examination

Eyes were examined with a stereomicroscope (SZ61, Olympus Corp., Tokyo, Japan) on days 4, 7, and 14 after alkali injury. The length of corneal neovessels was measured by using digital imaging software (analySIS TS, Olympus Corp., Tokyo, Japan). In order to visualize the corneal epithelium defects, we performed fluorescence staining of ocular surface using 0.5% fluorescein sodium salt (Sigma-Aldrich). The epithelial defect area was evaluated by using digital imaging software (analySIS TS, Olympus Corp.). Microscopic assessment was done by observers without prior knowledge of the procedures.

2.4. Histopathologic examination and immunohistochemical analysis

For histological observation of hematoxylin and eosin (H & E)-stained corneal sections by light microscopy (BX-51, Olympus Corp.), eyes were fixed in 10% phosphate-buffered formalin, routinely processed, and then embedded in paraffin. Tissue sections (6 μm) were prepared using a microtome (HM-340E, Thermo Fisher Scientific Inc., MA, USA) and placed on glass slides. H & E staining was performed according to standard techniques. The length of corneal epithelial thickness was measured by using digital imaging software (analySIS TS, Olympus Corp.). The proportion and extent of CNV were determined as previously described [5]. Most sections were taken from the central region of the cornea and CNV was evaluated in at least two sections from each eye.

For the detection of macrophages and CCR2 expression, immunohistochemical staining was conducted as previously reported [6]. In brief, corneal tissue sections were incubated with rat anti-mouse F4/80 antibody (eBioscience, San Diego, CA, USA) or rabbit anti-mouse CCR2 antibody (Abcam, Cambridge, UK) overnight at 4 °C. Negative control slides were incubated with non-immune immunoglobulin of the same isotype under the same conditions. The sections were further incubated with biotin-conjugated rat or rabbit Ig secondary antibody. The immune complexes were detected using a DAB Substrate Kit from Vector Laboratories according to the manufacturer's instructions. Finally, the tissues were counterstained with hematoxylin and mounted. The numbers of positive cells per square millimeter was counted in five randomly selected fields on the immunohistochemically stained slides at 200-fold magnification. Images were analyzed using a light microscope (BX-51, Olympus Corp.) and digital imaging software (analySIS TS, Olympus Corp.).

2.5. Murine peritoneal macrophage isolation and cell culture

Specific pathogen-free 8 week-old female mice were injected intraperitoneally with 1 ml of sterile 3% thioglycolate medium (Sigma-Aldrich), and macrophages were harvested 3 days later as described previously [21]. The cells were suspended in RPMI 1640 medium (Thermo Fisher Scientific Inc., Waltham, IL, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific Inc.), 100 IU/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific Inc.). The cells were incubated in a humidified incubator at 37 °C with 5% CO_2 in 24-well cell culture plates. Two hours later, nonadherent cells were removed and the medium was replaced. The cells were then stimulated with the indicated concentrations of ZER for 24 h with or without 1 μg/ml LPS (Sigma-Aldrich). The human corneal fibroblast cell line was kindly provided by Dr. James V. Jester (University of California, Irvine, CA). Cells were cultured in Dulbecco's Modified Eagle medium (DMEM; Thermo Fisher Scientific Inc.) with high glucose, 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 incubator. The cells were stimulated in the same conditions as mentioned above.

2.6. Cell viability assay

Cells were seeded in a 96-well plate (1×10^5 cells/ml) with 100 μl media in each well, and then allowed to adhere and grow for 24 h, followed by treatment with the indicated concentrations of ZER. After 24 h, cell viability was evaluated using the MTS assay (Promega, Madison, WI, USA). The absorbance of the samples was quantified at 490 nm using a spectrophotometer (EMax, Molecular Devices, Sunnyvale, CA, USA).

2.7. Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from tissue using the Easy-Spin Total RNA extraction kit (*GeneAll*, Seoul, *Korea*). Following incubation with