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Full Length Research Paper

Effect of tetrandrine on the expressions of hypoxia-inducible factor-1 and vascular endothelial growth factor in neovascularized cornea

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This study investigated the effect of tetrandrine on the expressions of hypoxia inducible factor1 (HIF1) and vascular endothelial growth factor (VEGF) in neovascularized cornea of rats following alkaline burn. A rat model corneal neovascularization (CNV) was established by alkaline burn. The expressions of HIF1 and VEGF before and after tetrandrine treatment were detected by immunohistochemistry and reverse transcriptase polymerase chain reaction (RT- PCR) and slit-lamp biomicroscopy and photography were done to observe the neovascularization. Result showed the expressions of HIF1 and VEGF in the normal cornea were very weak or even absent. Following alkaline burn, the expressions of HIF1 and VEGF were markedly increased and mainly located in the neovascularized area and in the epithelial layer. After treatment with Tetrandrine, the density of neovascularization was significantly decreased, accompanied by remarkably decreased expressions of HIF1 and VEGF in the neovascularized corneal stroma. It is concluded that tetrandrine can effectively inhibit the neovascularization following alkaline burn in rats and reduce the expressions of HIF1 and VEGF in neovascularized cornea of rats following alkaline burn.

Key words: Tetrandrine, corneal neovascularization, hypoxia inducible factor-1 , vascular endothelial growth factor.

INTRODUCTION

Normal cornea has no blood vessels. As a response to infection, trauma, immune reaction, rejection and other pathologic conditions, excessive in growth of blood vessels occurs from the limbal vascular plexus into the cornea, which is also known as CNV. CNV has been one of main causes of visual impairment and a risk factor for rejection following corneal transplantation (Benelli et al., 1997; Sun and Zhang, 2006). Nowadays, no effective treatments have been developed to prevent the CNV.

VEGF has been considered as a key regulator of CNV following hypoxia (Li et al., 2011). Recent study showed the activation of VEGF expression is dependent on HIF-1 which is required for the maintenance of its messenger ribonucleic acid (mRNA) stability (Büchler et al., 2003). Moreover, HIF-1 can also up-regulate the VEGF receptor which then promotes the angiogenesis. The present study was to investigate the effect of Tetrandrine (Tet) on the expressions of HIF-1 and VEGF in the n neovascularized rat cornea following corneal burn, which may provide a new strategy for the treatment of CNV in clinical practice.

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Abbreviations: HIF1 , Hypoxia inducible factor1 ; **VEGF,** vascular endothelial growth factor; **CNV,** corneal neovascularization; **mRNA,** messenger ribonucleic acid; **RNA,** ribonucleic acid; **RT-PCR,** reverse transcriptase polymerase chain reaction; **EPO,** erythropoietin; **PLA2,** phospholipase A2.

MATERIALS AND METHODS

Modeling and grouping

A total of 30 healthy Sprague-Dawley rats weighing 200~250 g were

purchased from the Experimental Animal Center of Tongji Medical College and randomly divided into 3 groups. In normal group (Group A; n=5), the cornea was untreated and served as negative controls. In the remaining 25 rats, alkali burn was introduced to all eyes. The right eyes served as positive controls and did not receive treatment (Group B) . The left eyes were treated with Tet (Group C). The alkali burn was prepared according to previously reported (Zhou et al., 2005): rats were anesthetized intraperitoneally with 10% chloral hydrate (3 mg/kg) and then ocular surface anesthesia was prepared with 0.25% tetracaine eye drops. The filter paper (3 mm in diameter) was immersed in 1 mol/L NaOH for 1 min and then placed centrally on the mouse cornea for 20 s. The alkali-treated cornea was then irrigated with normal saline for 1 min. The right eyes were treated with normal saline and the left eyes with Tet drops of designed concentrations (4 times/d) + 1% atropine eye ointment (1 time/d). This study has been approved by the Ethics Committee of The First Affiliated Hospital Affiliated to Zhengzhou University.

Preparation of Tet solution

Tet was purchased from Zhejiang Jinhua Pharmaceuticals (Powder, purity >98%). Tet was dissolved in normal saline at the concentrations of 5, 10, 20, 40 and 80 mg/kg. In our preliminary study, the effectiveness of Tet at 40 mg/kg was favorable. Thus, 40 mg/kg Tet was used in the present study as a treatment.

Quantification of CNV

At 1 day after alkali burn, slit lamp microscopy was performed to observe the in growth of blood vessels in the cornea of different groups and representative photographs were obtained. The length of newly generated blood vessels (continuous, less curved and vertical to the limbal tangent) was measured and the area with CNV was calculated according to the following equation: $S = C/12 \times 3.1416 \times (r^2-(r-l)^2)$ where S represents the area with CNV, C represents clock hours of neovascularization (1 clock hour equals 30 degrees of arc), r represents corneal radius and l represents length of newly generated blood vessels (Seo et al., 2001).

Sample preparation

1, 4, 7, 14 and 21 days after alkali burn, the whole cornea was collected and divided into two. One was fixed in 4% paraformaldehyde at 4°C for 24 h and embedded in paraffin followed by H and E staining and immunohistochemistry. The other part was stored in liquid nitrogen for reverse RT-PCR.

Immunohistochemistry for HIF1 and VEGF

Immunohistochemistry was performed with SP method. The primary antibodies were rabbit anti- rat VEGF polyclonal antibody (1:50) (Beijing Zhongshan Golden Bridge Biotech) and rabbit anti-rat HIF1 antibody (1:50) (Jingmei Biotech) and secondary antibody was biotin conjugated goat anti-rat IgG. In the negative controls, the primary antibody was replaced with PBS and the remaining procedures were similar to those above. The sections were observed under a light microscope and representative photographs were captured.

Detection of mRNA expression of VEGF by RT-PCR

Total ribonucleic acid (RNA) was extracted with TRIZOL according to manufacturer's instructions and the concentration and purity of RNA were determined by ultraviolet spectrophotometry. Then, 2 g of total RNA were subjected to reverse transcription with ReverTraAce- - TM kit followed by amplification of VEGF gene and -actin gene as an internal reference. The primers for VEGF were derived from previously report (Kompella et al., 2003).forward: 5'-TGGATCCATGAACTTTCTGCTGTC-3', reverse: 5'-TCACCGCCTTGGCTTGTCACAT-3'. These primers can be used for the amplification of two isomers of VEGF (VEGF165 and VEGF121) and the sizes of products were 584 bp and 452 bp, respectively. The primers for -actin were as follows: forward: 5'-AACCCTAAGGCCAACCGTGAAA-3', reverse: 5'-TCATGAGGTAGTCTGTCAGGTC- 3', and the anticipated size was 265 bp (Gohji et al., 2000). After gel electrophoresis, the bands of VEGF and -actin were photographed and the expressions of VEGF and -actin quantified.

Statistical analysis

The length of newly generated blood vessels, area with CNV and the mean optical density in immunohistochemistry were expressed as means ± standard deviation ($\bar{x}$ ±s). Statistical analysis was performed with SPSS version 11.0 statistic software package. Independent sample t-test was used to compare the difference between groups. A value of $P<0.05$ was considered statistically significant.

RESULTS

Ingrowth of blood vessels in the cornea

In normal corneas (Group A), blood vessels were not found. In Group B, 7 days after burn, the ingrowth of blood vessels was active and reached the area with burn. The mean length of blood vessels was 1.72 ±0.06 mm. In Group C, the in growth of blood vessels was markedly inhibited at 7 days after burn when compared with Group B, and the mean length of blood vessels was 0.89 ±0.04 mm. In the Group B and C, the mean area with CNV was 14.98 ±0.22 mm2 and 9.67 ±0.35 mm2, respectively. The length of newly generated blood vessels in the Group C was markedly shorter than that in the Group B (t l= 36.3979, P l< 0. 01, n = 10) and the area with CNV in the Group C was also dramatically smaller than that in the Group B (t s=40.6185, P s< 0. 01, n =10).

Immunihistochemistry for HIF-1 and VEGF

The evaluation of expressions of HIF-1 and VEGF was carried out according to previously reported (Pichiule et al., 1999) . HIF-1 is predominantly expressed in the cytoplasm and nucleus and VEGF in the cytoplasm or on the cell membrane. In the present study, HIF-1 expression was not found in the normal corneal. VEGF was not expressed in the cornea or weakly expressed in the epithelial basement membrane. In Group B, HIF-1 and VEGF expressions were observed in the inflammatory cells of stroma and the endothelial cells of

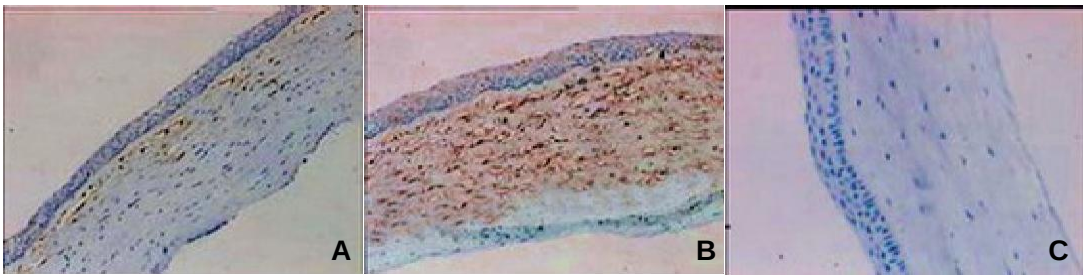

Figure 1. A. HIF-1 expression in normal cornea: No HIF-1 expression was found in the cornea of normal rats. B. HIF-1 expression in the Group B: The HIF-1 was highly expressed in the newly generated blood vessels of corneal stroma and the epithelium layer; C. HIF-1 expression in the Group C: the HIF-1 expression in the area with CNV was markedly reduced when compared with that in the Group B, which was consistent with the decrease of the density of blood vessels (immunohistochemistry, ×200).

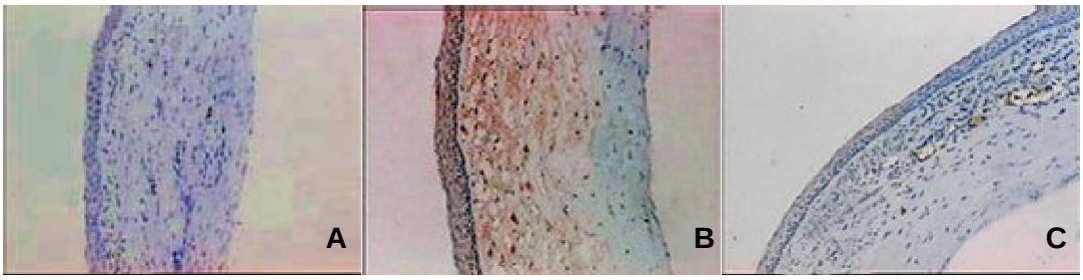

Figure 2. A. VEGF expressions in normal cornea: VEGF was weakly expressed in the epithelial basement membrane of normal rats. B. VEGF expression in the Group B. The VEGF was highly expressed in the newly generated blood vessels of corneal stroma and the epithelium layer; C. VEGF expression in the Group C: the VEGF expression in the area with CNV was markedly reduced when compared with that in the Group B, which was consistent with the decrease of the density of blood vessels (immunohistochemistry, ×200).

newly generated blood vessels at different stages. In the Group C, the expressions of HIF-1 and VEGF were markedly attenuated when compared with those in the Group B. The staining intensity and proportion of positive cells were significantly reduced (Figures 1 and 2).

VEGF mRNA expression

VEGF gene has different splicing products. In the present study, the primers can be used to amplify two VEGF isomers: VEGF121 (452 bp) and VEGF165 (584 bp). Results showed the mRNA expression of VEGF was very low in normal cornea. In the Group B, the mRNA expression of VEGF was markedly increased at the early stage and returned normal 7 days after alkali burn. In the Group C, after Tet treatment, the mRNA expression of VEGF was only slightly increased when compared with that in normal cornea (Figure 3 showed the VEGF expression 4 days after burn). In Group A, B and C (4 days after burn), the mRNA expression of VEGF was 0.258±0.069, 1.293±0.211 and 0.512±0.093, respectively. Statistical analysis showed the VEGF level in the Group B was markedly higher than that in the Group A (t =14.7434, $P < 0.01$) and the Group C (t = 10.7107, $P < 0.01$).

DISCUSSION

The initiation and development of CNV are regulated by a series of factors in which VEGF plays an important role. Studies have found that hypoxia is a critical cause of up-regulation of VEGF which has been considered as a key regulator of CNV following hypoxia (Chaoran et al., 2011; Li et al., 2011; Mochimaru et al., 2008). In the CNV following ocular ischemia, the involvement of VEGF is necessary. In the in vitro endothelial cells undergoing hypoxia, the mRNA and protein expressions of VEGF were markedly increased. HIF-1 is a transcription factor ubiquitously existing in the mammals and humans and consists of two subunits (HIF-1 and HIF-1). HIF-1 is an oxygen sensitive subunit and can regulate the expressions of multiple target genes including glucose transporters, VEGF, erythropoietin (EPO), etc. It has been demonstrated that HIF-1 is crucial for the maintenance of energy metabolism in the cancer cells, the stimulation of angiogenesis and promotion of the proliferation and migration of cancer cells (Paradziej-Lukowicz et al., 2011).

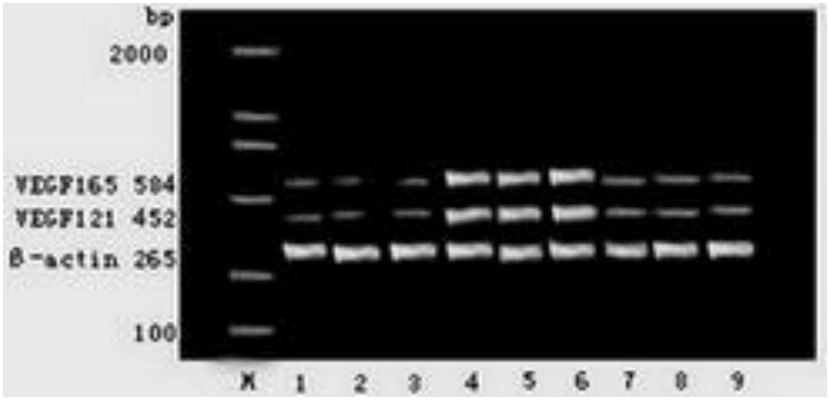

Figure 3. mRNA expression of VEGF in the cornea: Lane 1~3: Group A Lane 4~6: Group B; Lane 7~9: Group C (after Tet treatment).

Recent study showed the activation of VEGF expression is dependent on HIF-1 which is required for the maintenance of its mRNA stability (Büchler et al., 2003). Moreover, HIF-1 can also up-regulate the VEGF receptor which then promotes the angiogenesis. Tet is a kind of alkaloids isolated from the root of Stephania tetrandra and a double benzylisoquinoline derivative. It has multiple pharmacological activities including anti-inflammatory, analgesia, antipyretic, antiallergenic, anti-free radical and anti-tumor effects. Tet had therapeutic effects on the experimental uveitis and keratitis. *In vivo* study revealed Tet could significantly inhibit the rabbit posterior capsule opacification and the membrane formation on the surface of implanted intraocular lenses, suppress the lipid peroxidation in the anterior chamber following surgery and attenuate the damage to local eye tissues.

In vitro experiments also demonstrated the growth and proliferation of rabbit skin fibroblasts, conjunctival fibroblasts and retinoblastoma cells could be markedly inhibited by Tet treatment. Our results showed Tet could exert suppressive effect on the CNV which may be related to the attenuation of the damage by free radicals. The potential mechanisms may be as follows: 1). Tet can block the influx of extracellular calcium. It is currently accepted that calcium overload in the cytoplasm and mitochondria may facilitate the lipid peroxidation which results in damage to membrane structure and in turn leads to calcium overload in the cytoplasm and mitochondria forming a vicious circle. Tet can decrease the intracellular calcium concentration of newly generated blood vessels, which reduced the production of free radicals induced by intracellular calcium overload. 2). Tet can inhibit the phospholipase A2 (PLA2), lipid peroxidation and subsequent production of free radicals. Tet could inhibit the activity of PLA2 in a dose dependent manner (He et al., 1995). The suppression of PLA2 not only reduces the lipid peoxidation and production of free radicals but decrease the production of PGE2, LTB4 and PAF which are important vasoactive substances. Both effects are contributed to the therapeutic effects of Tet on CNV. 3).

Tet can inhibit the synthesis and release of inflammatory factors and then reduce the production of oxygen free radicals. PLA2 and other inflammatory mediators together with the damage to the endothelial cells can activate platelets leading to release of oxygen free radicals and subsequent damage to cornea. Experiments revealed Tet could inhibit the synthesis of both PGF2 and LTB4 (Li et al., 1999), and suppress the production of interleukin- 1 by monocytes and macrophages and TNF- by lymphocytes (Seow et al., 1992). Moreover, Tet can also reduce the synthesis of PAF. Through decreasing the pro-inflammatory mediators including prostaglandins, leukotrienes, and interleukins, Tet reduces the production of oxygen free radicals exerting anti-free radical effect. We speculated that Tet had anti-free radical activity and could improve the hypoxia following alkali burn reducing the expression of HIF-1 . The VEGF expression is mainly regulated by the HIF -1 under hypoxia conditions, which is also critical for the maintenance of mRNA stability. The reduced expression of HIF- 1 was consistent with the decrease of VEGF expression following Tet treatment. Our results revealed Tet could improve the CNV via reducing the expressions of HIF- 1 and VEGF following alkali burn in a rat model exerting therapeutic effects. Thus, Tet may become an adjuvant alternative for the treatment of CNV.

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