·Basic Research·

β-catenin expression in rat neovascularized cornea after alkali burn

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Abstract

• AIM: To investigate the expression of β -catenin in cornea after alkali burn and explore its role in cornea neovascularization (CNV).

• METHODS: CNV model was established by putting filter paper with the size of 3.0mm in diameter immersed in 1mol/L NaOH solution on the left cornea of rat for 20 seconds. Twenty-five Sprague Dawley rats were randomly divided into 5 groups: post-operation 1-, 4-, 7-, 14- and 21-day groups while the right eyes as normal control group. The expression level of β -catenin protein, mRNA and VEGF were determined at the 1st, 4th, 7th, 14th and 21st day following the establishment of model by RT-PCR and immunohistochemical technique.

• RESULTS: No expression of β -catenin immunoreactivity was detected in normal cornea. The expressions of β -catenin and VEGF both reached the peak at the 4th and 7th day and gradually decreased to near baseline 21 days later. Alteration of β -catenin and VEGF levels showed a significant positive correlation (r=0.855, P<0.05).

• CONCLUSION: The levels of β -catenin are markedly related to inflammatory CNV in rat cornea after alkali burn.

• KEYWORDS: β -catenin; VEGF; cornea alkali burn; corneal neovascularization

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INTRODUCTION

Corneal neovascularization (CNV) is a common pathological condition, which is a major cause of blindness and is a high risk factor for graft rejection after allograft corneal transplantation. CNV can be affected by multiple agents, and vascular endothelial growth factor (VEGF) is one of the most significant factors in angiopoiesis. VEGF can alter the permeability of the vascular endothelial cell (VEC), thus to cause the migration and proliferation of the VEC. Recent research showed that β-catenin in *wnt* pathway participates in angiopoiesis in tumorigenesis and inflammation, by regulating the expression of VEGF, which is a target gene downstream[1]. On account of the importance of β-catenin in angiopoiesis, and the relationship between β-catenin and VEGF, we detected the expression of β-catenin and VEGF in CNV, in order to describe the mechanisms of CNV, and accordingly investigate new methods to inhibit it.

MATERIALS AND METHODS

Materials Twenty-five healthy Sprague Dawley (SD) rats of random gender (200-250g), aged 2-3 months were obtained from the Experimental Animal Science Center of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). The rats after alkaline burn in left eyes were randomly divided into 5 groups: post-operation 1-, 4-, 7-, 14-, and 21-day groups while the right eyes as normal control group. The rats were anesthetized by intraperitoneal injection with 100g/L chloral hydrate (3mL/kg). The alkaline burn was created in the left eye of each rat by contacting the central area of the cornea surface with a 3.0-mm-diameter circular filter disc saturated with 1mol/L NaOH for 20 seconds. Cornea and conjunctival sac were then irrigated with 20mL physiological saline immediately for one minute. NaOH was replaced by physiological saline in the right eye of each rat.

Methods From the first day after cornea alkali burn, measured and serial photographs of the cornea were taken under the slit-lamp biomicroscope. CNV was quantified by calculating the wedge-shaped area of vessel growth with the formula: $A=C/12\times3.1416[r^2-(r-l)^2]$[2], where A is the area, C is time (in hours), r is the radius of the cornea, and l is the length of new vessels. Five animals of each group were sacrificed at day 1, 4, 7, 14, and 21 after observation and measurement of CNV areas, and each experimental cornea

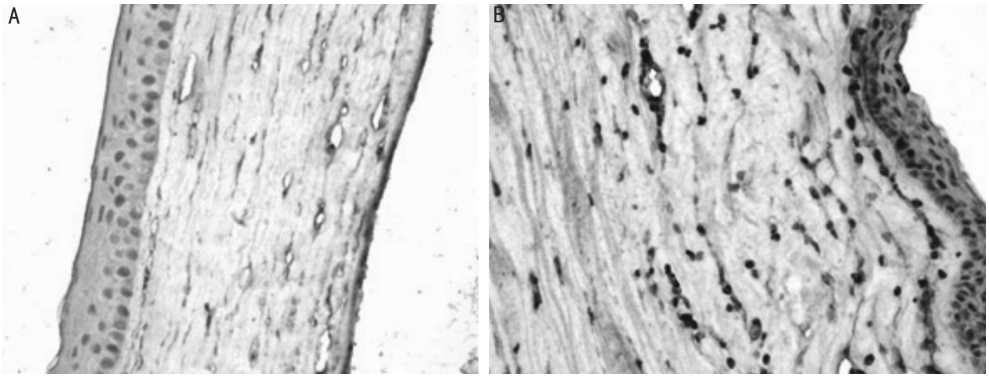

Figure 1 VEGF expression in rat cornea (SP×400) A: Control; B: 4 days after alkali burn

was divided into two halves. One half was placed into 40g/L paraformaldehyde phosphate buffer for immunohistochemistry analysis. The other half was homogenated on ice immediately and stored in -80℃ Trizol for extraction of total RNA of the cornea in RT-PCR analysis. According to the SP routine method of immunohistochemical staining technique, the fixed cornea tissue was immersed into graded ethanol to get dehydrated, and then immersed into xylene to get transparent. The tissue was immersed into liquid paraffin at 56℃, embeded in paraffin, and sliced into serial sections. Finally immuno- histochemical staining was performed on 4mm thick paraffin sections following the kit instruction (provided by Wuhan Boster Company), meanwhile in the normal controls, we replaced the primary antibody with PBS. All the slides were examined under microscope and photographed after thorough washing. Gray scale of rat cornea represented the expression of β-catenin was examined using a HMIAS-2000 image analysis system with five random highpower fields of every sheet.

Total mRNA was extracted from frozen tissue (the RT-PCR kit and PCR reagents were provided by Wuhan Lingfei Technology Co. Ltd). The published sequence of the primers for the amplification of β-catenin and the housekeeping gene β-actin were as follows respectively [3,4]: forward 5'-GCT GAC CTG ACG GAG TTG GA-3' reverse 5'-GCT ACT TGC TCT TGC GTG AA-3' (the length of production was 227bp), and forward 5'-CTG GAA GGT GGA CAG TGA G-3'reverse 5'-GAG GGA ATT CGT GCG AGA C-3' (the length of production was 665bp). Electrophoresis was carried out after PCR product was extracted. The absorbance (A) of the bands specific for each β-catenin or β-actin was quantified using Sensi An sys gel image analysis, and the proportionality of β-catenin and β-actin absorbance, as relative intergral absorbance (RIA), represents the relative expression of β-catenin mRNA in each group. The expression of VEGF was detected using immunohistochemical staining technique, described as above.

Statistical Analysis All the data were statistically evaluated by analysis of variance, and t test and correlation analysis were performed with SPSS Version 13.0 for Windows, $P<0.05$ was considered significant.

RESULTS

Biomicroscopic findings Twenty-four hours after alkaline burn, only epithelium hydropsia was observed, and vessels in the limbus of the cornea were congested. At day 4, new vessels began to grow from the limbus to the transparent cornea. Seven days after alkali burn, the growth rate of CNV areas reached its peak, and the branches were obviously observed and anastomosed with each other. CNV area maximum was seen at day 14, and the vessels were interlaced to each other. Thenceforward, the areas of CNV began to diminish, some vessels became thinner, and several those were degenerated.

VEGF Expression Immunohistochemistry analysis (Figures 1A and 1B) revealed that one day after alkali burn, there were a number of inflammatory cells infiltrating in substantia propria layer. The lumens of CNV became to emerge since the 4th day, and expression of VEGF was detected in endothelial cell of CNV and in substantia propria layer. Image analysis showed that the content of VEGF protein began to rise since the 1st day after alkali burn, reached its peak at day 4, started to decrease at day 7, and decrease significantly after 14 days. Yet there was no expression of VEGF in normal cornea tissue. The statistical analysis showed that the expression of VEGF (gray value) in 1-, 4-, 7- and 14-day group *vs* control group was higher and there was a statistical significance between these post-operation groups and control group (t_{1d}=12.407, t_{4d}=29.439, t_{7d}=16.048, t_{14d}=13.317, $P<0.05$). However, the increased expression of VEGF in 21-day group was not obvious, and there was no statistical significance (t_{21d}=1.595, $P>0.05$).

β -catenin Expression There was no expression of β-catenin in normal corneal tissue. However, the immunocompetence of β-catenin began to enhance at day 1 after alkali burn, and peaked at day 4, which mainly located

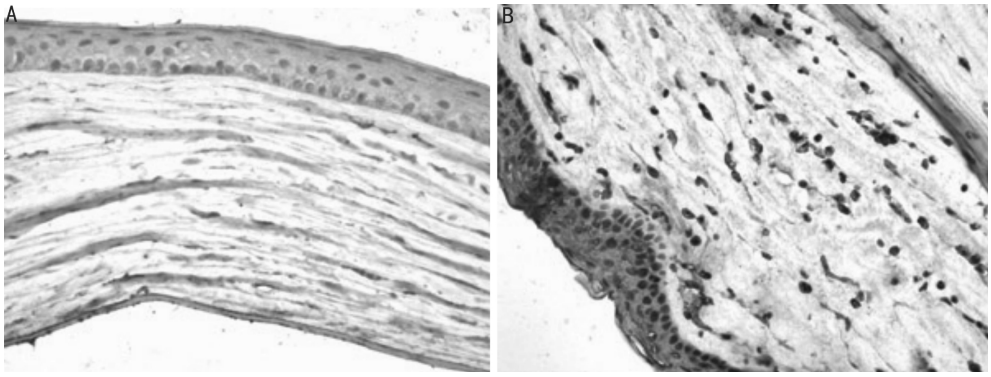

Figure 2 β−catenin expression in rat cornea (SP×400) A: Control; B: 4 days after alkali burn

on epithelial lamina and substantia propria layer. It started to decrease after day 14, and fell down to normal level at day 21 (Figures 2A and 2B). According to the statistical analysis, the tendency of β-catenin expression was in positive correlation with VEGF (coefficient correlation r=0.855, P< 0.05). The electrophoresis images of RT-PCR (Figure 3) showed that there was a low expression of β-catenin mRNA in normal cornea tissue, and began to increase right after the cornea alkali burn. The expression of β-catenin reached the highest level during day 4 to day 7, and began to decrease after that till it recuperated to normal level after day 21. Compared to the control group, the expression of β-catenin in day 1, 4, 7 and 14 were obviously higher. There was statistical significance between these post-operation groups and control group (t_{1d}=16.081, t_{4d}=21.779, t_{7d}=21.526, t_{14d}= 4.79, P<0.05), meanwhile the expression in day 21 group were not higher enough and there was no statistical significance (t_{21d}=1.479, P>0.05).

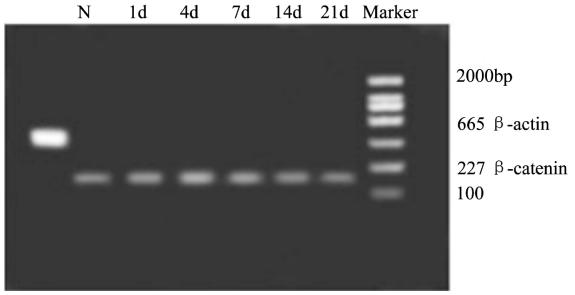

Figure 3 β−catenin gene and β−actin gene in rat cornea (RT−PCR)

DISCUSSION

β-catenin is a cellular protein with multiple functions. As an important component of the adherens junction complex, it helps to anchor E-cadherin to the intracellular actin cytoskeleton [4]. Meanwhile, as an important *wnt* signal transducer, β-catenin plays an important role in many developmental processes. In normal case, cellular nomadic β-catenin is keeping being degradated due to GSK23β, thus it maintains at a rather low level [5]. When extracellular signaling molecules are integrated with membrane receptors, GSK23β kinase gets inactivated, which induces accumulation of intracellular β-catenin and then ingression to cell nucleus through nucleopore[6].

Previous researches have manifested that β-catenin can alter the cell cycle of epithelium, which consequently could promote epithelium proliferation and survival, and could augment angiogenesis by inducing the expression of VEGF and activating vascular ancestral cell. Kim *et al* [7] discovered that the human vascular endothelial growth factor (VEGF) gene promoter has been reported to contain binding sites for β-catenin/Tcf, and the transfection of β-catenin to normal colon epithelial cells significantly increased VEGF expression. Massod *et al* [8] also revealed that the members of VEGFs, such as VEGF-A, VEGF-C and VEGFR-2 were down stream of targets genes of the *Wnt* pathway, and β-catenin could strengthen the activity of them. As a key member in *Wnt* signaling pathway, which has the transcriptional control activity, β-catenin can combine with another member Tcf/Lef, so to activate the transcription of the target genes involved in cell proliferation and survival in cell nucleus. Accordingly, activated β-catenin is capable of activating neovascularization effectively *via* multiple ways. Our study revealed that from day 1 after alkali burn, the immunocompetence of β-catenin started to enhance, and it peaked at day 4, decreased after day 14, and finally fell down to normal level at day 21. The electrophoresis images of RT-PCR showed that the expression of β-catenin began to increase right after the cornea alkali burn, reached the highest level during day 4 to day 7, and it began to decrease after that till it recuperated to normal level after day 21. According to the statistics analysis, alteration of β-catenin and VEGF levels showed a significant positive correlation (r= 0.855, P<0.05).

Our experiment proved that the levels of β-catenin were markedly related to inflammatory corneal neovascularization in rat cornea after alkali burn. That suggests more methods

could be provided for the cure of corneal neovascularization by inhibiting or intervening the activity of β-catenin.

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