·Basic Research·

COX -2 and its inhibitor Celecoxib in corneal neovascularization

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Abstract

• AIM: To observe the expression of COX-2 in rat corneal neovascularization (CNV) and its relationship to CNV, and to explore the inhibition of Celecoxib, a COX-2 inhibitor, to CNV.

• METHODS: The distribution and quantification of COX-2 and VEGF was detected by immunohistochemistry. Expression of COX-2 and VEGF mRNA was quantified by RT-PCR. The difference in protein and mRNA expressions of COX-2 and VEGF was analyzed to find the correlation between them.

• RESULTS: Expression of activated COX-2 and VEGF protein and mRNA in CNV had a dynamic change. VEGF and COX-2 co-localized. Compared with the control group, expression of both protein, mRNA of COX-2 and VEGF in experimental group II and III had significant difference($P<0.05$),indicating the correlation between COX-2 and VEGF, while that in experimental group I had no statistical difference ($P>0.05$).

• CONCLUSION: COX-2 expression was up-regulated in inflammatory CNV. COX-2 modulates the expression of VEGF, playing a very important role in CNV. Celecoxib inhibit COX-2 expression so as to hold back the CNV.

• KEYWORDS: corneal neovascularization; VEGF; COX-2; celecoxib

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INTRODUCTION

Cyclooxygenase (prostaglandin endoperoxides, COX) is the rate-limiting enzyme in prostaglandin (PG) synthesis, which has two isoenzymes: COX-1 and COX-2. COX-1 participates in physiological function, while COX-2 takes part in many pathophysiological processes, such as inflammation, injury, repair, neovascularization, tumor growth and metastasis. In this research, we focused on the mechanism of COX-2 in corneal neovascularization (CNV) and the inhibition effect of Celecoxib, a high selective COX-2 inhibitor, on CNV, providing a theoretical foundation for CNV treatment in clinic.

MATERIALS AND METHODS

Materials Sprague Dawley (SD) rat (Experimental Animal Science Center of Tongji Medical College, Huazhong University of Science and Technology); Celecoxib(Mol Wt : 381.373, Mol Fm:C17H14F3N3O2S, Sigma); rabbit anti-rat COX-2 polyclonal antibody, rabbit anti-rat VEGF monoclonal antibody, SP immunohistochemistry kit (Wuhan Boster Company); RNA Extract kit, RT kit, PCR kit and Tween-20 (Wuhan Lingfei Technology). Primer synthesis (Shanghai Yingjun Bioengineering).

Methods

Induction of CNV Forty SD rats with 80 eyes were anesthetized with 100mL/L chloral hydrate intraperitoneal injection. 2mm-diameter filter paper was saturated with 1mol/L NaOH. Redundant fluid was discarded by using sterilized cotton buds. After a conjunctival sac wash with 1∶2 000 gentamicin in normal saline, a piece of filter paper was placed gently on the central cornea and left for 25 seconds. The filter paper was then removed, and cornea and conjunctival sac were washed with normal saline for 1 minute. After the treatment, rats were housed in separate cages on a standard 12/12-h light-dark cycle, with water and food pellets available ad libitum.

Animal groups and drug administration CNV model rats were randomly divided into four groups: experimental group I, II, III, and control group. Rats in each group received 1, 2, 3μ mol/L Celecoxib solution, and normal saline subconjunctival injection respectively. The administration was performed everyday in the first week and every other day in the second week.

Observation of morphology Condition of corneal opacity, CNV and conjunctival congestion were observed in a slit lamp microscope at 1,2,4,7,14 days after alkali burn. Length and amount of CNV developed from limbus corneae was measured and photographed. The longest but least continuous curvature CNV towards the center of corneal opacity was recorded as L. The area of CNV ($A = C/12 \times 3.14 \times [r^2 - (r-L)^2]$) was determined by measuring with the cornea radius (r), the vessel length (L) from the limbus, and the number of clock hours (C) of limbus involved.

Immunohistochemistry At 1,2,4,7,14 day after alkali burn, the rats were deeply anesthetized and sacrificed, and then the eyes were removed. The right eyes were immediately placed in a refrigerator and kept at -70℃; corneas of the left eyes were dissected at 1mm from the limbus. After fixation, paraffin imbedding, sectioning, the pathology of alkali burn was detected through HE staining. Some other sections were stained by SP method using rabbit anti-rat COX-2 polyclonal antibody as well as rabbit anti-rat VEGF monoclonal antibody as primary antibodies, and biotinylated goat anti-rabbit IgG as secondary antibody. Positive control was the set of rat kidney instead of cornea, and the negative controls were reactions using PBS instead of primary antibody. COX positive staining showed brown at nuclear membrane and plasma. Only a blue nuclear was determined as negative. VEGF positive cells exhibited brown granules in plasma. Integration optic density (IOD) of COX-2 and VEGF in rat cornea was examined using a HMIAS-2000 image analysis system. Average and standard deviation of IOD in each group was calculated.

COX –2 and VEGF mRNA expression in cornea by RT –PCR The rat corneas which kept in the refrigerator were reserved for the use of RT-PCR. The RNA extracts were obtained by one-step method. Optic density (OD) of total RNA was measured in an ultraviolet spectrophotometer at a wave length of 280/260nm to calculate total RNA concentration. cDNA was synthesized according to the guideline in the kit, 70℃ 5 minutes, 42℃ 5 minutes, 37℃ 60 minutes, 70℃ 10 minutes. A sense primer 5'-ACACTC TATCACTGGCATCC-3' and a reverse primer 5'-GAAGG GACACCCTTTCACAT-3' for COX-2 produced 561bp sequences[1], and a sense primer 5'-AGCCCATGAAGTGGT GAA -3' and a reverse primer 5'-TGCGGATCTTGGACAA AC-3' for VEGF generated 383bp sequences. Internal control β-actin product, 207bp, was amplified using a sense primer 5'-CACCCGCGAGTACAACCTTC-3' and an adverse primer 5'-CCCATACCCACCATCACACC-3'. The PCR products were separated on a 15g/L ethidium bromide-stained agarose gel. The strands were visualized in a Gel-Pro analyzer. Data were analyzed with BIO2RAD Quality One software. Intergroup comparison was performed through a ratio of COX-2 to β-actin and VEGF to β-actin.

Statistical Analysis The relationship of COX-2 and VEGF expression between each experimental group and control group was analyzed by variance analysis, linear t-tests, and interclass correlation for statistical analysis with SPSS12.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Morphology At 1 day after Alkali burn, vessels in limbus of cornea were congested and cornea center was edema and not clear. At 2 day, new vessel began to grow from limbus to transparent cornea. The growth rate of CNV was the fastest from 2 day to 4 day. After 7 days, the growth rate began to slow down. In 14 days, CNV grow slimmer, some of which degenerated. Celecoxib had inhibitor effect in CNV at different time-point. CNV in control group and experimental group II 4 days after alkali burn was observed (Figure 1,2). Under light microscope, superficial substantia propria layer in experimental group II had lighter edema and inflammatory cell infiltration *vs* control group, new vessels in experimental II were in smaller amount, with slimmer lumens and less arrangement (Figure 3,4).

Immunohistochemistry Cornea epithelium in normal rat expressed weak COX and VEGF. In control group, after alkali burn cornea epithelium showed COX-2 and VEGF positive at each period. At 1 day after alkali burn, cornea epithelium expressed COX-2 and VEGF. At 2 day, cornea epithelium and substantia propria layer had more inflammatory cell filtration with COX-2 and VEGF positive, filtrating into canal-like structure in substantia propria layer. Endothelium in vessel wall had positive staining. At 4 day, strong expressions of COX-2 and VEGF could be detected. Mature CNV lumens could be seen in substantia propria layer. Endothelium was positive stained (Figure 5). At 7 day, inflammatory cells decreased in substantia propria layer, where contained larger diameter and compact arrange capillary. CNV endothelium was COX-2 and VEGF positive. At 14 day, COX-2 and VEGF expression in cornea significantly declined and capillary in substantia propria layer decreased, too. There was a weaker expression of

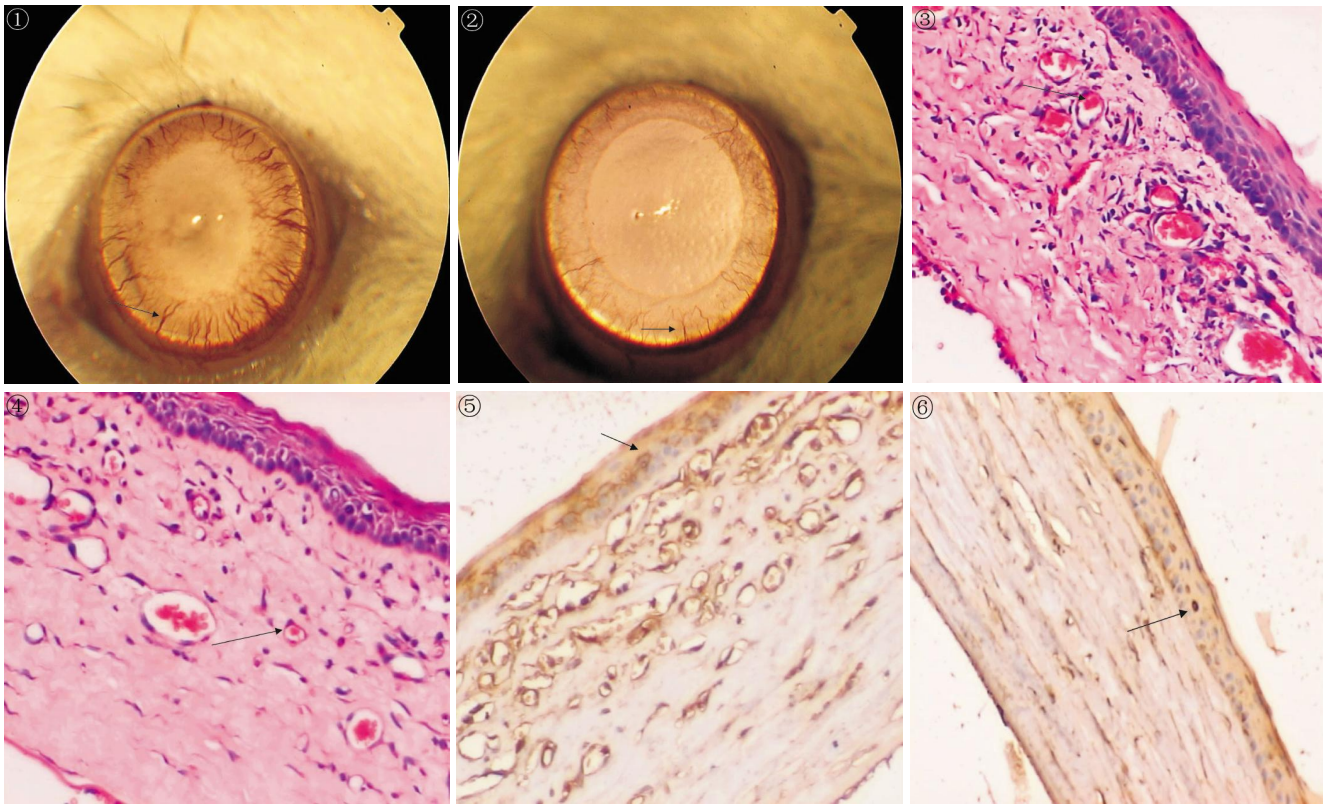

Figure 1 CNV in control group 4 days after alkali burn. The new vessels were densely and gross, the cornea was limous

Figure 2 CNV in experimental group II 4 days after alkali burn. The new vessels were sparse and slender, the cornea was translucent

Figure 3 Pathology in control group 4 days after alkali burn. There was more inflammatory cell filtration in cornea epithelium and substantia propria layer, new vessels were in a large amount, with gross lumens and coarctate arrangement

Figure 4 Pathology in experimental group II 4 days after alkali burn. There was less inflammatory cell filtration in cornea epithelium and substantia propria layer, new vessels were in a small amount, with narrow lumens and rare arrangement

Figure 5 Immunohistochemistry in control group 4 days after alkali burn. Strong expressions of COX-2 could be detected, mature CNV lumens could be seen in substantia propria layer. Endothelium was positive stained

Figure 6 Immunohistochemistry in experimental group II 4 days after alkali burn. Very weak expression of COX-2 in epithelium and substantia propria layer. No evident mature CNV lumens has be seen in substantia propria layer

COX-2 and VEGF in cornea epithelium, inflammatory cells, and CNV endothelium in group II and III than that in control group (Figure 6, P =0.008, P =0.007). The difference in group I and control was not significant (P = 0.998). COX-2 and VEGF co-localized in the same region. At each period, COX-2 and VEGF in experimental groups and control group had significant direct correlation (r = 0.943, P=0.000).

RT-PCR The weak expressions of COX-2 and VEGF mRNA turned to increase at 1day after alkali burn, peaked at 4 day, declined at 7 day, and remained weak expression at 14 day (Figure 7). A ratio of COX-2 and VEGF in experimental group I and control group had no difference. The expressions of COX-2 and VEGF mRNA decreased in group II and III when compared with control group (Figure 8). The expressions of COX-2 and VEGF mRNA in experimental groups and control group had significantly direct correlation (r =0.945, P=0.000), the same with immunohistochemistry.

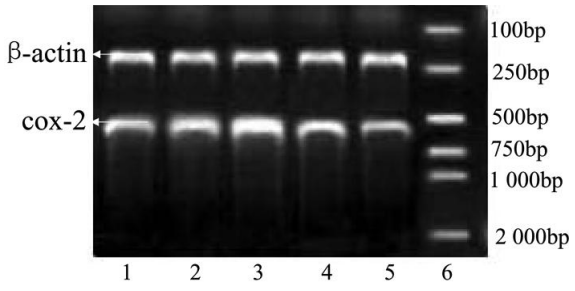

Figure 7 COX-2 mRNA expression in control group at each period after alkali burn. 1-5 represent the different time-point after alkali cautery, 6 : marker. The expressions of COX-2 mRNA turned to increase 24 hours after alkali burn, peaked at 4 day, declined at 7 day, and remained weak expression at 14 day

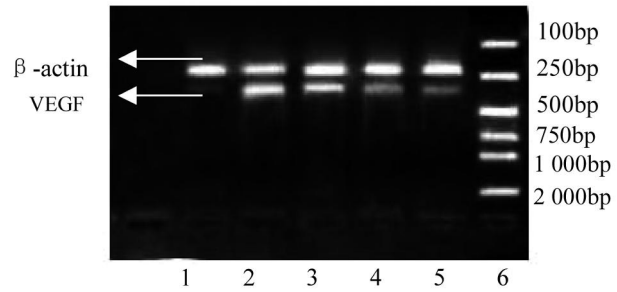

Figure 8 VBGF mRNA expression in experimental group and control group 4 days after alkali burn. The expressions of VEGF mRNA was very weak in normal cornea. It decreased in experimental group II and III when compared with control group, without significant difference between experimental group I and control group

DISCUSSION

CNV is a crucial reason in inflammatory keratopathy inducing decreased vision and corneal graft rejection. COX-2 is an important inducible enzyme in inflammation. This enzyme usually does not express in normal condition, but was stimulated or induced by anoxia, growth factors, mediators of inflammation, tumor derivatives, and oncogene. Main physiological function of COX-2 is to catalyze the transformation of arachidonic acid to PG, a potential mechanism in CNV advancement. VEGF is the most specific and direct vascular endothelial cell stimulating factor, which directly binds surface receptor on epithelium cell advancing the angiogenesis [2]. Cianch *et al* [3] found the immunohistochemical expressions of both COX-2 and VEGF in colorectal carcinogenesis were significantly increased and correlated with microvessel density. PGE2 levels were significantly higher in metastatic tumors than in nonmetastatic ones. VEGF is related to angiogenesis and hereby one of the most important mediators of COX-2 angiogenic pathway. Using genetic and pharmacological techniques, Williams *et al* [4] reported a 94% reduction in COX-2 (-/-) mouse fibroblasts' ability to produce the proangiogenic factor, VEGF, and a 92% reduction in VEGF with the treatment of wild-type mouse fibroblasts with a selective COX-2 inhibitor, suggesting COX-2 modulates angiogenesis through the regulation of VEGF synthesis. In animal experiment, Majima *et al* [5] demonstrated that COX-2 inhibitor can inhibit neovascularization in sponge granuloma. In addition, some experiments also proved that COX-2 inhibitor suppressed TXA2 production, endothelial migration, and fibroblast growth factor-induced corneal angiogenesis [6], and that the angiogenesis can be inhibited only by nonspecific cyclooxygenase inhibitors or COX-2 inhibitors, not by COX-1 inhibitors. All these evidence indicates COX-2 is closely related with angiogenesis.

Non-steroid drugs like indometacin and Brufen are nonspecific cyclooxygenase inhibitors, showing inhibition to CNV and potential side effect. Products of COX-1 may play an important role in keeping the normal proliferation of corneal epithelium. So the inhibition of COX-1 may impair the proliferation and migration of normal corneal epithelium and the healing of damaged epithelium, impeding the recovery of keratopathy. Celecoxib is a high selective COX-2 inhibitor. The inhibition to COX-2 is 375 times stronger than COX-1 [7]. Hence, therapeutic dose celecoxib avoids such disadvantage. Surya *et al* [8] reported that in a streptozotocin-induced diabetic rat model celecoxib inhibits the expressions of retina COX-2 and VEGF as well as reduces diabetes-induced retinal VEGF mRNA expression to normal level.

Our study induced CNV model by alkali burn, a well duplication of chemical burn induced CNV disease. Surya *et al* urther found the bioavailability is 52 folds higher following subconjunctival injection compared with peritoneal injection [9, 10] . The drug concentration in ipsolateral cornea and sclera is nearly the same. The drug educes effects mainly by infiltration on the injection site. In our research, we set a concentration gradient in subconjunctival injection to optimize the drug concentration. We found expressions of COX-2, VEGF protein and mRNA began to increase 1 day after alkali burn, peaked at 4 day, declined at 7 day, and significantly decreased at 14 day. The expressions of COX-2 and VEGF had correlation. After local celecoxib administration, the expressions of COX-2 and VEGF in experimental group II and III significantly declined compared with control group, but it had no difference in group II and III as well as group I and control group. So we concluded that subconjunctival injection of 2μmol/L celecoxib had significant inhibitive effect on alkali burn induced CNV, providing a clue to prevent CNV.

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