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Ketorolac inhibits choroidal neovascularization by suppression of retinal VEGF

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Abstract

We assessed the effect of topical ketorolac on laser-induced choroidal neovascularization (CNV), measured retinal PGE₂ and VEGF levels after laser treatment, and determined the effect of ketorolac on PGE₂ and VEGF production. Six laser burns were placed in eyes of rats which then received topical ketorolac 0.4% or artificial tears four times daily until sacrifice. Fluorescein angiography (FA) was performed at 2 and 3 weeks and retinal pigment epithelium-choroid-sclera flat mounts were prepared. The retina and vitreous were isolated at 1, 3, 5, 7, and 14 days after laser treatment and tested for VEGF and PGE₂. Additional animals were lasered and treated with topical ketorolac or artificial tears and tested at 3 and 7 days for retinal and vitreous VEGF and PGE₂. Ketorolac reduced CNV on FA by 27% at 2 weeks ($P < 0.001$) and 25% at 3 weeks ($P < 0.001$). Baseline retina and vitreous PGE₂ levels were 29.4 $\mu\text{g/g}$ and 16.5 $\mu\text{g/g}$ respectively, and reached 51.2 $\mu\text{g/g}$ and 26.9 $\mu\text{g/g}$ respectively, 24 h after laser treatment ($P < 0.05$). Retinal VEGF level was 781 pg/g 24 h after laser treatment and reached 931 pg/g by 7 days ($P < 0.01$). Ketorolac reduced retinal PGE₂ by 35% at 3 days ($P < 0.05$) and 29% at 7 days ($P < 0.001$) and retinal VEGF by 31% at 3 days ($P = 0.10$) and 19% at 7 days ($P < 0.001$). Topical ketorolac inhibited CNV and suppressed retinal PGE₂ and VEGF production.

Keywords

age-related macular degeneration; AMD; choroidal neovascularization; CNV; laser-induced CNV; nonsteroidal anti-inflammatory drugs; NSAIDs; ketorolac; cyclooxygenase; COX-2

1. Introduction

Choroidal neovascularization (CNV) is a leading cause of severe vision loss in patients with age-related macular degeneration (AMD), posterior uveitis (Kedhar et al., 2007), pathologic myopia (Soubrane, 2008), and ocular histoplasmosis (Cohen et al., 1996). Although the pathogenesis of CNV is complex, a growing body of scientific evidence indicates that

Conflict of Interest

No conflicting relationship exists for any author.

cyclooxygenase (COX) plays a contributory role (Kim and Toma, 2010; Takahashi et al., 2004; Yanni et al., 2009).

Cyclooxygenase (COX) is an important enzyme in the inflammatory process and catalyzes the biosynthesis of eicosanoids from membrane derived arachidonic acid to produce prostaglandins (PGs) and thromboxanes. PGs have wide ranging effects within the eye including vasodilation, disruption of the blood-ocular barrier, and leukocyte migration. Accumulating evidence indicates that PGs also promote CNV (Kim et al., 2010). In direct support of this, COX can be detected in human choroidal neovascular membranes (Maloney et al., 2009) and inhibition of COX in a variety of experimental systems suppresses choroidal neovascular growth (Sakamoto et al., 1995; Takahashi et al., 2004; Hu et al., 2005).

Vascular endothelial growth factor (VEGF) is a principle mediator of CNV and PGs interact with and amplify VEGF (Hatazawa et al., 2007). In vitro studies have demonstrated that PGE₂ increases VEGF expression in cultured rat Muller cells (Cheng et al., 1998) and agonism or antagonism of the PGE₂ receptor EP₄ increases or decreases VEGF production respectively, in a dose-dependent manner (Yanni et al., 2009).

Ketorolac is a potent nonsteroidal anti-inflammatory drug (NSAID) and inhibits COX enzymes and thereby the synthesis of PGs. Moreover, it is commercially available in topical formulations (Acular[®] LS, Allergan, Irvine, CA; Acuvail[™], Allergan, Irvine, CA) for ophthalmic use. In order to further clarify the role of COX and PGs in CNV, we assessed the effect of topical ketorolac on experimental laser-induced CNV, characterized the temporal relationship of retinal PGE₂ and VEGF levels after laser photocoagulation, and determined the effect of COX inhibition by ketorolac on retinal PGE₂ and VEGF production.

2. Methods

All procedures were performed with strict adherence to guidelines for animal use and experimentation set forth by the Vanderbilt University Animal Care and Use Committee and the Association for Research in Vision and Ophthalmology.

2.1. Animal groups and treatments

Adult male Brown Norway rats (Charles River Laboratories, Inc, Wilmington, MA), approximately 6 weeks of age, were selected for all experiments in this study and maintained in a controlled environment with a 12-h on/off light cycle. Food was available *ad libitum*. For all procedures, animals were anesthetized by an intramuscular injection of ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (5 mg/kg). Pupils were dilated with phenylephrine (2.5%) and atropine sulfate (1%). Proparacaine (0.5%) was applied for corneal anesthesia.

2.2. Laser photocoagulation

Laser photocoagulation was performed using a method similar to that described by Edelman and Castro (Edelman and Castro, 2000). In brief, animals were positioned before a slit lamp (Carl Zeiss Meditec, Jena, Germany) laser delivery system. The fundus was visualized using a microscope slide coverslip with 2.5% hydroxypropyl methylcellulose solution as an optical coupling agent. An argon green laser (Coherent, Santa Clara, CA) was used for photocoagulation (532 nm wavelength; 360 mW power; 0.07 s duration; 50 μ m spot size). This setting most reliably produced acute vapor bubbles indicative of the rupture of Bruch's membrane. In each eye, focal laser was applied concentrically approximately two optic discs from the center avoiding major blood vessels.

2.3. Topical ketorolac vs artificial tears (control)

In each eye of 12 rats, six focal laser applications were performed. Immediately afterwards, six rats (12 eyes) were treated with topical ketorolac 0.4% (Acular® LS) and six rats (12 eyes) were treated with topical artificial tears (Optive™, Allergan, Irvine, CA) four times daily until sacrifice. The volume of each drop was approximately 50 µl. Both eyes of each animal received the same treatment to avoid the possibility of a crossover effect.

2.4. Assessment of CNV response to treatment

To quantify longitudinal response to treatment, animals underwent fluorescein angiography (FA) on days 14 and 21 after laser treatment. For FA evaluations, 0.3 ml of 10% sodium fluorescein was administered intraperitoneally and photographs of the right and left eye were taken. Angiograms consistent with early-mid phase (approximately 4–6 min after injection of fluorescein) were used for all comparative analysis.

On day 21 after FA imaging, animals were euthanized by cervical dislocation and eyes were enucleated and stored in 10% formalin for 2 h. Retinal pigment epithelium (RPE)-choroid-sclera (choroidal) flat mounts were prepared as previously described.¹³ In brief, the cornea and lens were removed and after dissecting the retina from the eyecup and discarding it, radial cuts were made in all four quadrants in order to flatten the remaining tissue. The flattened RPE-choroid-sclera tissue was then mounted in Gel Mount (Biomedica; Victoria, Australia). Endothelial cells were identified using FITC-conjugated *Bandeiraea simplicifolia* isolectin B4, (Sigma-Aldrich, Inc., St. Louis, MO) and the elastin of the surrounding extracellular matrix was stained using goat anti-elastin antibody conjugated to Cy3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Choroidal flat mounts were visualized using the 10× objective of an epifluorescent compound microscope fitted with the appropriate excitation and emission filters (Provis AX-70, Olympus, Japan). Images of the neovascular lesions were captured using a digital camera attached to the Provis system (DP71, Olympus, Japan) coupled to a computer with image capture software (DP Controller, Olympus, Japan).

CNV staining and leakage on FA and vascular budding on choroidal flat mounts were calculated by measuring the pixel area within the circumference of each rupture site using Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, CA). In brief, CNV lesions on FA were measured by a trained, masked investigator, using the “quick selection tool” in Adobe Photoshop CS3 to measure the pixel area contained within the best-fitting polygon. On choroidal mounts, the pixel area of vascular budding was traced by a trained, masked investigator using the “lasso” selection tool in Adobe Photoshop. The cross-sectional area of each CNV lesion was quantified as the area contained within the best-fitting polygon.

2.5. PGE₂ and VEGF levels after focal laser photocoagulation

To determine the time course of PGE₂ and VEGF expression in the retina and vitreous after focal laser photocoagulation, each eye of 10 rats received eight focal laser applications. At 1,3,5,7, and 14 days after laser treatment, the retina and vitreous from 2 animals (4 eyes) were isolated and placed immediately in passive lysis buffer (Promega, Madison, WI) for 1 h and then frozen immediately at –80 °C for later testing. In brief, a longitudinal incision was made into the cornea and the lens–vitreous complex was extracted en bloc. The retina was then dissected from the eyecup using fine forceps. The lens–vitreous complex was placed on a 600 micron filter (Safar Nitex, Heiden, Switzerland) and centrifuged at 20,000 g over a pre-weighed microcentrifuge tube for 1 min to isolate the vitreous from lens material. VEGF and PGE₂ protein concentration were measured using the mouse VEGF-164 ELISA kit (R&D Systems; Minneapolis, MN) and PGE₂ kit (R&D Systems; Minneapolis, MN) respectively, according to the manufacturer’s instructions. The amount of VEGF and PGE₂

in retina and vitreous was normalized to the weight of the tissue in grams (g). Four additional rats (8 eyes) were not lasered and served as baseline controls.

2.6. PGE₂ and VEGF levels after treatment with ketorolac vs artificial tears

Each eye of 24 rats received eight focal laser applications. After laser treatment, both eyes of 12 rats received topical ketorolac and 12 rats received artificial tears 4 times daily until sacrifice. The retina and vitreous from 6 animals in each group were isolated at 3 and 7 days after laser treatment and prepared and tested for VEGF and PGE₂ as described above.

2.7. Statistics analysis

Results were expressed as mean $\pm$ 95% confidence intervals. Comparison of mean values was performed using an unpaired Student's *t*-test with unequal variance. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Fluorescein angiography and choroidal mounts

A total of 6 rats (12 eyes) were treated with ketorolac and 6 rats (12 eyes) were treated with artificial tears after laser photocoagulation. No eyes were excluded due to lens trauma or severe vitreous bleeding. Laser rupture sites which had subretinal bleeding at the time of lasering were excluded from analysis and represented less than 10% of total rupture sites in each treatment group.

Early-mid phase FA images at 2 and 3 weeks demonstrated consistent and visually detectable differences in rupture site staining and leakage between eyes treated with ketorolac versus artificial tears (Fig. 1). Topical ketorolac significantly reduced CNV leakage on FA by 27% at 2 weeks ($P < 0.001$) with an average CNV lesion size of 6711 ± 528 versus 9184 ± 915 pixels for eyes treated with artificial tears (Fig. 2). This inhibitory effect of ketorolac remained significant at 3 weeks. Mean CNV lesion size at 3 weeks for ketorolac and artificial tear-treated eyes was 6973 ± 461 and 9238 ± 950 pixels, respectively ($P < 0.001$). There was minimal progression in lesion size between 2 and 3 weeks for both treatment groups.

Choroidal flat mounts at 3 weeks demonstrated a visibly detectable reduction in vascular budding in ketorolac treated eyes compared to artificial tears (Fig. 3). Topical ketorolac significantly ($P < 0.001$) reduced vascular budding on choroidal flat mounts with a mean lesion size of $19,9205 \pm 13,640$ pixels ($191 \pm 6 \mu\text{m}^2$ area) versus $24,4386 \pm 29,522$ pixels ($210 \pm 13 \mu\text{m}^2$ area) for eyes treated with artificial tears (Fig. 4).

3.2. PGE₂ and VEGF levels after laser photocoagulation

Baseline PGE₂ concentrations in the retina and vitreous were $29.4 \mu\text{g/g}$ and $16.5 \mu\text{g/g}$ respectively and reached a maximum concentration of $51.2 \mu\text{g/g}$ and $26.9 \mu\text{g/g}$ respectively 24 h after laser treatment ($P < 0.05$, Fig. 5). PGE₂ concentration in the retina and vitreous declined to $12.8 \mu\text{g/g}$ and $13.8 \mu\text{g/g}$ respectively by 3 days and remained less than baseline levels, but not statistically different, at all subsequent time points measured.

Baseline VEGF concentration in the retina was 922 pg/g compared to 781 pg/g 24 h after laser treatment ($P < 0.01$, Fig. 6). Retinal VEGF concentration increased after 24 h and reached a maximum concentration of 931 pg/g by day 7, which was significantly greater than day 1 VEGF levels ($P < 0.01$), but not significantly different from baseline levels ($P = 0.69$). In contrast to retinal VEGF, vitreous VEGF concentration was significantly greater than baseline at all time points measured after laser treatment. Baseline vitreous VEGF

concentration was 26 pg/g and reached a maximum concentration of 101 pg/g ($P < 0.001$) at 5 days. Vitreous VEGF concentration remained significantly elevated at 14 days compared to baseline ($P < 0.01$).

3.3. PGE₂ and VEGF levels after treatment with ketorolac vs artificial tears

Three days after laser photocoagulation, ketorolac treatment significantly reduced retinal PGE₂ by 35% ($P < 0.05$) with an average concentration of $12.0 \mu\text{g/g} \pm 4.3$ versus $18.5 \mu\text{g/g} \pm 3.6$ for eyes treated with artificial tears (Fig. 7). Ketorolac treated eyes also showed a non-significant 9% reduction in vitreous PGE₂ concentration. At 7 days after laser treatment, ketorolac treatment significantly reduced retinal PGE₂ by 29% ($P < 0.001$) and vitreous PGE₂ by 11% ($P < 0.05$) with a average concentration of $9.5 \mu\text{g/g} \pm 0.4$ and $20.1 \mu\text{g/g} \pm 0.9$, respectively versus $13.5 \mu\text{g/g} \pm 0.3$ and $22.6 \mu\text{g/g} \pm 1.7$, respectively for eyes treated with artificial tears.

Three days after laser treatment, ketorolac reduced retinal VEGF by 31% with an average concentration of $596 \text{ pg/g} \pm 130$ versus $866 \text{ pg/g} \pm 253$ for eyes treated with artificial tears, but this difference was not statistically significant ($P = 0.10$, Fig. 8). By 7 days, however, ketorolac significantly reduced retinal VEGF by 19% ($P < 0.001$) with an average concentration of $978 \text{ pg/g} \pm 48$ versus $1201 \text{ pg/g} \pm 58$ for eyes treated with artificial tears.

At both 3 and 7 days after laser treatment, vitreous VEGF concentrations were not significantly different between ketorolac- and artificial tear-treated groups.

4. Discussion

The results of our in vivo study suggest that inhibition of PGE₂ production by ketorolac reduces laser-induced CNV at least in part by suppressing retinal VEGF expression. Furthermore, our results show that prostaglandin levels in the retina peak at 24 h after laser rupture of Bruch's membrane and retinal VEGF levels peak at 5–7 days post-laser, establishing a temporal relationship between these 2 mediators and providing further support of a cause and effect relationship. To our knowledge, we are the first to report that topical ketorolac 0.4% (Acular® LS) inhibits CNV and suppresses retinal PGE₂ and VEGF levels.

Cyclooxygenase (COX) is an important enzyme in the inflammatory process and catalyzes the biosynthesis of eicosanoids from membrane-bound arachidonic acid to produce prostaglandins (PGD₂, PGE₂, PGF₂), prostacyclin (PGI₂), and thromboxane (TXA₂). Two isoforms of COX, COX-1 and COX-2, are firmly established. COX-1 is constitutively expressed in almost all tissues and is responsible for normal housekeeping functions. In contrast, COX-2 expression is induced by a wide variety of pathologic and physiologic stimuli. In the human retina, COX-2 has been shown to be the predominant isoform in human RPE and is significantly up-regulated by pro-inflammatory cytokines (Chin et al., 2001).

In vitro studies have shown that PGE₂ directly stimulates VEGF expression in cultured rat Muller cells (Cheng et al., 1998) and selective agonism and antagonism of the PGE₂ receptor EP₄ increases and decreases VEGF production, respectively, in a dose-dependent manner (Yanni et al., 2009). Furthermore, selective inhibition of COX-2 prevents VEGF expression in cultured human RPE cells (Amrite et al., 2006). Previous studies have shown that inhibition of COX-2 suppresses VEGF expression in trauma-induced and ischemia-induced animal models (Takahashi et al., 2003; Yanni et al., 2009), but to our knowledge this is the first study to demonstrate similar suppression of VEGF by COX-2 inhibition in experimental laser-induced CNV.

It is now firmly established that VEGF is the principle mediator of ocular angiogenesis and is up-regulated by hypoxia in retinopathy of prematurity and proliferative diabetic retinopathy. While retinal hypoxia from thickening of Bruch's membrane impeding oxygen exchange from the underlying choriocapillaris may contribute to increased VEGF expression in AMD, there are several clinical conditions such as ocular histoplasmosis and punctate inner choroidopathy where CNV frequently occurs in the apparent absence of tissue hypoxia. In these later conditions, local inflammation and inflammatory mediators may be the predominant pathway for VEGF expression (Lee et al., 2009).

In this regard, laser-induced CNV is an appropriate model to further investigate the relationship of inflammation and VEGF expression. A growing body of recent scientific work supports the role of inflammation in CNV (Rodrigues, 2007; Patel and Chan, 2008). Genetic analysis in human AMD patients suggest that the pathogenesis of up to 50% of cases of AMD may be explained by a polymorphism (loss of function) of complement factor H, which serves to down-regulate the complement system (Haines et al., 2005; Klein et al., 2005). The complement system comprises over 30 soluble and membrane-bound proteins that initiate pro-inflammatory responses. More direct evidence comes from histological studies which demonstrate the arrival of macrophages at laser rupture sites within 1 h after laser application (Miller et al., 1990) and subsequent accumulation of complement components C3a, C5a, and C5b-9 (Bora et al., 2005). Complement components are found in drusen (Patel and Chan, 2008), recruit inflammatory cells, and promote CNV (Nozaki et al., 2006). Furthermore, macrophages are present in histologic specimens taken from patients with AMD and gather in areas of disruption of Bruch's membrane (Lopez et al., 1991; Grossniklaus et al., 2002).

Treatment of human RPE cells with inflammatory cytokines, TNF- α and IL-1 β , increases PGE₂ production by 9-fold and 250-fold respectively (Chin et al., 2001) and both cytokines are secreted by macrophages in response to tissue injury. Furthermore, selective pharmacologic inhibition of TNF- α or IL-1 β inhibits CNV in a dose-dependent fashion (Olson et al., 2007; Olsen et al., 2009).

Consequently, we propose the following sequence of events that may occur after laser photocoagulation. Laser rupture of Bruch's membrane results in immediate recruitment of macrophages and other inflammatory cells within the first hour of tissue injury (Miller et al., 1990) which secrete TNF- α and IL-1 β among other pro-inflammatory cytokines. TNF- α and IL-1 β upregulate COX-2 expression (Chin et al., 2001) in neighboring RPE cells which increases production of PGE₂ achieving peak retinal levels by 24 h. Via both autocrine and paracrine pathways, PGE₂ increases VEGF expression in RPE and/or other resident retinal cells (Yanni et al., 2009) and retinal VEGF levels increase steadily until approximately 7 days. Compromise of the physical barrier of Bruch's membrane (Yu et al., 2008) in conjunction with increased retinal VEGF promotes choroidal angiogenesis. Resolution of the inflammatory response by intrinsic down-regulatory mechanisms results in declining levels of VEGF by 14 days and subsequent stabilization of choroidal neovascular complexes by 10–14 days. Spontaneous involution occurs after 30 days as has been previously described (Edelman and Castro, 2000).

This proposed mechanism ties together our study findings with the findings of others and explains the mechanism of CNV inhibition observed by many anti-inflammatory agents (NSAIDs, corticosteroids, anti-TNF- α , anti-IL-1 β). Such a mechanism is consistent with clinical observations that patients taking aspirin or other anti-inflammatory agents appear to have a reduced incidence of neovascular AMD (Wilson et al., 2004; Kim et al., 2010) and that CNV occurs at specific locations in ocular inflammatory conditions such as punctate inner choroidopathy where inflammation is thought to be present.

Consistent in three separate experiments, we were surprised to observe a decrease in retinal VEGF levels from baseline at 24 h after laser treatment. However after 24 h, retinal VEGF levels rose steadily until day 7 as expected and reported by others (Yu et al., 2008). A possible explanation for our observation is early emigration of VEGF from the retina into the much larger vitreous space prompted by compromise of tissue and vascular integrity by laser photocoagulation. This could result in decreased retinal VEGF levels early on until steady state is achieved. In addition, our findings are in contrast to Takahashi et al. who reported inhibition of CNV with topical nepafenac but not with ketorolac (Takahashi et al., 2003). However, upon closer review of their published work, there was a trend favoring ketorolac inhibition of CNV when compared to vehicle that may have become significant with a larger sample size. Furthermore, their experiments were conducted on C57BL/6 J mice not Norway Brown rats and thus their results are not directly comparable to ours. Future work investigating the direct relationship of COX enzymes, prostaglandin receptors, and VEGF production in wild type and COX-2^{-/-} cell lines would be informative.

In conclusion, topical ketorolac significantly inhibited CNV via suppression of retinal PGE₂ and VEGF expression. The results of this study further support the role of COX and prostaglandins in CNV and suggest a potential therapeutic role for NSAIDs.

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Fig. 1. Representative fluorescein angiogram images of laser-induced choroidal neovascularization (CNV) at 2 and 3 weeks.

Fig. 2. Mean pixel area of CNV lesions measured on fluorescein angiogram at 2 and 3 weeks after treatment with ketorolac or artificial tears. Error bars represent 95% confidence intervals (CI). * $P < 0.00001$. ** $P < 0.0001$.

Fig. 3. Representative choroidal flat mounts demonstrating laser-induced CNV and degree of vascular budding in eyes treated with ketorolac or artificial tears at 3 weeks.

Fig. 4. Mean pixel area of CNV lesions measured on choroidal flat mounts at 3 weeks after treatment with ketorolac or artificial tears. Error bars represent 95% CI. * $P < 0.00001$.

Fig. 5. Mean PGE₂ concentration in the retina and vitreous before and after laser-induced choroidal neovascularization (LCNV). PGE₂ concentration at 1 day after LCNV was significantly greater than baseline in both the retina and vitreous. * $P < 0.05$.

Fig. 6. Mean VEGF concentration in the retina and vitreous before and after LCNV. Compared to day 1 after LCNV, retinal and vitreal VEGF concentrations reached their highest level at 7 days ($P < 0.01$) and 5 days ($P < 0.001$) respectively.

Fig. 7. Retina and vitreous PGE₂ concentration at 3 and 7 days after LCNV. Three days after laser treatment, ketorolac significantly reduced retinal PGE₂ by 35% ($P < 0.05$) compared to eyes treated with artificial tears. At 7 days, ketorolac significantly reduced retinal PGE₂ by 29% ($P < 0.001$) and vitreous PGE₂ by 11% ($P < 0.05$).

Fig. 8.

Retina and vitreous VEGF concentration at 3 and 7 days after LCNV. At 3 days, ketorolac reduced retinal VEGF by 31% compared to eyes treated with artificial tears ($P = 0.10$). At 7 days, ketorolac significantly reduced retinal VEGF by 19% ($P < 0.001$). At both 3 and 7 days vitreous VEGF concentrations were not significantly different between ketorolac and artificial tear-treated groups.