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To cite this article: S Altan, H Sağsöz & Z Oğurtan (2017): Topical dimethyl sulfoxide inhibits corneal neovascularization and stimulates corneal repair in rabbits following acid burn, Biotechnic & Histochemistry, DOI: 10.1080/10520295.2017.1371333

To link to this article: https://doi.org/10.1080/10520295.2017.1371333

Published online: 13 Dec 2017.

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Topical dimethyl sulfoxide inhibits corneal neovascularization and stimulates corneal repair in rabbits following acid burn

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Abstract

Neovascularization of the cornea is characterized by the growth of blood vessels caused by imbalances between angiogenic and anti-angiogenic factors. We investigated whether the expression of Vascular endothelial growth factor (VEGF), Vascular endothelial growth factor receptor (VEGF), Vascular endothelial growth inhibitor (VEGI) receptors, as well as topical drug treatments, participate in regulating corneal neovascularization after corneal damage and remodeling. We used 72 mature male New Zealand rabbits. Corneal burns were induced by hydrofluoric acid under general anesthesia. The rabbits were treated with indomethacin or dimethyl sulfoxide (DMSO). The animals were euthanized on days 2, 7 and 14 after injury. Each cornea was fixed with 10% neutral formalin. On days 2, 7 and 14, VEGF, flk1/KDR and flt1/fms were strongly expressed in the epithelial, stromal and inflammatory cells, but not in the corneal endothelial cells. On day 7, newly formed blood vessels were observed growing toward the center of the cornea. In the control, indomethacin treated, DMSO treated, and indomethacin + DMSO treated animals, VEGI, VEGF, and the receptors, flk1/KDR, flt1/fms and flt4, were expressed at different densities in the neovascular regions. This was particularly evident in the indomethacin- and indomethacin + DMSO-treated groups on days 7 and 14, compared to day 2. Treatment with VEGF and DMSO stimulated repair of corneal damage. We suggest that VEGI in the endothelial cells of neovascularized cornea may act as a signaling protein that promotes balance between cell proliferation and apoptosis. Topical administration of DMSO inhibited corneal neovascularization more effectively than indomethacin.

Key words: angiogenesis, burns, cornea, cytokine, dimethyl sulfoxide, DMSO, eye, hydrofluoric acid, indomethacin, neovascularization, rabbit, VEGI
vascular permeability (Chang et al. 2012). VEGF induces cellular responses by binding to the tyrosine kinase receptors located on the cell surface. These receptors include VEGFR-1 (flt1/fms; the fms-like tyrosine kinase), VEGFR-2 (flk1/KDR; the kinase insert domain-containing region), and VEGFR-3 (flt4) (Chang et al. 2001, 2012).

Vascular endothelial growth inhibitor (VEGI) is a specific inhibitor of angiogenesis. In most adult tissues, VEGI plays a physiological role in the balanced maintenance of vascularization (Metheny-Barlowa and Li 2006). In addition, VEGI may act as an autocrine factor that induces apoptosis in endothelial cells by activating multiple signaling pathways, such as those involving stress protein kinases and certain caspases (Yue et al. 1999). Previous studies of the cornea suggest that the administration of natural and synthetic inhibitors of angiogenesis may be an important strategy for regulating angiogenesis (Bock et al. 2007, Chang et al. 2001, Kim et al. 2010). We are aware of no reports, however, concerning the administration and expression of VEGI.

The development of CNV following chemical burns caused by exposure to acids and alkalis is a significant health problem for humans and animals (Cursiefen et al. 2000). Although severe damage may not occur immediately, severe organ damage eventually may occur if penetration of the chemicals is deep into the tissue and if these cases are untreated (Burgher et al. 2011). Because alkaline substances cause more severe eye damage, studies of chemical burns of the eye have focused on alkaline chemicals (Amano et al. 1998, Bock et al. 2007, Chang et al. 2001, Cursiefen et al. 2000, Gan et al. 2004, Kim et al. 2010, Onder et al. 2014, Ozdemir et al. 2014, Shi et al. 2011, Totan et al. 2001, Zhang et al. 2005).

Hydrofluoric acid (HF) is widely used industrially, e.g., in the glass, leather, petrochemical and semiconductor industries. It differs from other acids in its ready penetration of biological membranes owing to its lipophilic nature. Consequently, this acid can have marked effects on the health of humans and other species (Atley and Ridyard 2015). The principal goal in the treatment of HF burns is to enable re-epithelization of the damaged tissues by removing both the chemical exposure and the inflammatory products that have been generated (Wagoner 1997). To do this, the contact site is washed immediately with a nontoxic fluid, antibiotics and both steroid and non-steroid anti-inflammatory drugs (Prabhat and Sanaz 2007).

In experimentally induced cases of chemical corneal burns, the non-steroid drug, indomethacin, has been administered, either alone or in combination with antioxidants, as an alternative to steroids. Unlike steroids, indomethacin contributes to corneal and conjunctival re-epithelization by decreasing the production of prostaglandin E2 (PGE2) (Koay 1996). Dimethyl sulfoxide (DMSO) also has been used to treat chemical burns, because it possesses analgesic, anti-inflammatory, antimicrobial and antioxidant properties (Balicki 2012, Laria et al. 1997). DMSO also is characterized by low toxicity and good tissue permeability, and it facilitates the penetration of other substances through biological membranes. Therefore, it is not surprising that investigators have reported beneficial effects of DMSO. For example, DMSO drops were found to accelerate the healing of corneal chemical burns in rabbits (Skrypuch et al. 1987, Tozczołowski et al. 1992). Use of 50% aqueous DMSO, combined with dexamethasone to treat chronic superficial keratitis (CSK) in dogs was reported to be more effective for reducing the inflammation than the administration of dexamethasone alone (Balicki 2008). Long-term administration (10 months) of DMSO for treatment of CSK reduced the formation of inflammatory products and caused no side effects in the corneal epithelium.

Previous research has shown that for corneal chemical burns caused by alkaline substances, acute inflammation and neovascularization were associated with increased expression of VEGF and certain VEGF receptors (flk1/KDR) that are known to be significant angiogenic factors for corneal neovascularization (Ozdemir et al. 2014, Totan et al. 2001). We induced experimental burns of rabbit cornea using HF to establish a model of corneal neovascularization. We addressed several questions. First, are VEGI, VEGF, and VEGF receptors (flt1/fms, flk1/KDR and flt4, respectively) expressed by the corneal epithelial and stromal cells? Are these factors involved in corneal repair during topical application of DMSO and indomethacin? Are these factors involved in regulation of corneal neovascularization? Does topical administration of indomethacin and DMSO constitute an effective treatment for corneal neovascularization?

Material and methods

Animals

The Ethics Committee for Experimental Animals of Selcuk University approved all procedures used for our study; we also complied with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, USA (approval number 10/15). We used 72 6–8-month-old 2.0–2.5 kg adult male
New Zealand rabbits that were obtained from Sima Tavşancılık (Konya, Turkey). The animals were maintained with a 12 h light:12 h dark cycle and provided pellet feed and water ad libitum.

Chemicals and drugs

For all rabbits, acid burns of the corneas were caused by 2% HF (38–40% Merck, Darmstadt, Germany) applied to one eye; the other eye was used as a control. Treatment consisted of 40% DMSO in 0.9% saline, 0.1% aqueous indomethacin (5 ml Indocolir®; Abdil-Ibrahim, Istanbul, Turkey) or a combination of the two. The DMSO solution (99.9%, Merck) was prepared by mixing 40 volumes of pure DMSO with 60 volumes of 0.9% aqueous sodium chloride. The resulting solution was transferred into 5 ml sterile vials. A commercially available preparation, containing 0.1% aqueous indomethacin also was used. All groups were treated with a topical preparation of tobramycin to prevent infection. The animals were anesthetized with an aqueous solution containing 2% xylazine hydrochloride (Rompun®; Bayer, Istanbul, Turkey) and 10% ketamine hydrochloride (Ketasol®, Interhas, Istanbul, Turkey). The animals were given 14 mg/kg dipyrone (Devalgine®), Istanbul, Turkey) or a combination of dipyrone and ketamine. The DMSO and indomethacin solutions at 6 h intervals four times a day. Group 3 received four drops of a combination of DMSO and indomethacin solutions at 6 h intervals four times a day at doses equal to those administered to the groups that received DMSO and indomethacin alone. Group 4 was the control and received no treatment. The applications were made only to the right eye in all groups.

Evaluation of CNV

The pattern, size, and severity of CNV in each group was assessed using a hand-held slit-lamp microscope before and after staining with 1% fluorescein strips, and images of the eyes were captured using a digital camera. NV scores were assigned to each eye based on the distance from the limbus to the end point of the CNV, toward the central corneal burn, according to the procedure of Onder et al. (2014). NV scores were assigned as: 0, no vessels visible in the cornea; 1, NV extended 1/4 of the distance to the burn; 2, NV extended 1/3 of the distance to the burn; 3, NV extended 1/2 of the distance to the burn; 4, NV extended 2/3 of the distance to the burn; 4.5, NV extended 3/4 of the distance to the burn; 6, vessels reached the burn.

On days 2, 7 and 14 following application of HF, the rabbits were euthanized with an overdose of sodium pentothal. The treated eyes were excised, the corneal tissue was cut at its margin and divided in half. Both halves were fixed in 10% neutral buffered formalin for 24 h. After washing in water, dehydrating, and clearing, the tissue samples were embedded in paraffin and serial sections were cut at 5 µm. Five slides were prepared from each sample for immunohistochemical (IHC) analysis. Each slide held four sections to show the NV fields in different parts of the cornea. The clinical and histopathological changes following an HF-induced acid burn were described for all groups in our previous report (Altan and Ogurtan 2016).

Immunohistochemistry (IHC)

IHC analyses were performed using the streptavidin–biotin–peroxidase complex and the Zymed Histostain-Plus Bulk Kit (85–9043, Histostain Plus Bulk Kit, Zymed, San Francisco, CA). The serial sections were deparaffinized, rehydrated, and washed in distilled water. After each treatment, the sections were washed three times with 0.01 M phosphate buffered saline (PBS) for 5 min each. Endogenous peroxidase activity was blocked by immersing the sections in 3% v/v H2O2 in distilled water for 20 min. The sections were incubated in blocking serum for 15 min, then
The sections were incubated at 4°C with the primary antibody, as summarized in Table 1. The sections subsequently were treated with biotinylated secondary antibodies (Histostain Plus Bulk Kit; Zymed) and enzyme-conjugated streptavidin for 20 min (Zymed) and enzyme-conjugated streptavidin (Zymed) for 20 min. The sections were incubated in a diaminobenzidine (DAB) chromogen solution for 5–15 min to visualize the peroxidase antibody label. After counterstaining with Gill’s hematoxylin for 2 min, the sections were washed in running tap water until they acquired a blue color. The sections then were passed through a graded series of alcohols, xylene and embedded with Entellan.

The specificity of the IHC procedure was confirmed using positive and negative control sections. Sections of human breast carcinoma, colon and placenta processed as described above served as positive controls for immunoreactions to all antibodies. For negative controls, primary antibodies were replaced with PBS, normal rabbit IgG (sc-2027; Santa Cruz Biotechnology, San Francisco, CA, USA) or normal Mouse IgG (sc-2025; Santa Cruz Biotechnology,). All samples were subjected to the same protocol.

The sections from all experimental groups were examined and imaged using a Nikon Eclipse E400 (Nikon, Tokyo, Japan) microscope equipped with a digital camera (Nikon Coolpix 4500).

**Semiquantitative evaluations**

Immunohistochemical staining was evaluated semiquantitatively using the quick score (QS) double grading system, which consisted of the sum of an intensity score (IS) and a proportional score (PS) (Sagsoz et al. 2013). For this method, the intensity of staining (IS) is judged as follows: 0, negative; 1, weak staining; 2, intermediate staining; 3, strong staining. The proportion of stained nuclei also was recorded as 0, 0%; 1, approximately 1%; 2, 1–10%; 3, 11–33%; 4, 34–66%; 5, 67–100%. The score for intensity then was added to the score for proportion to give the quick score with a range of 0–8.

The evaluations of the positively stained cells were graded blindly by two observers (S. Altan and H. Sagsoz) and the mean scores were calculated. The expression of VEGF and its receptors as well as VEGI in the cornea was examined microscopically at 400×. For the peripheral and central cornea, five randomly selected areas were evaluated/section for specimens obtained on days 2, 7 and 14 of the experiment. The results for the different corneal layers are presented as: surface epithelium, stromal cells, inflammatory cells, and endothelial cells of Descemet’s membrane.

**Evaluation of vessel counts**

Positive immunoreactions for VEGF, its receptors and VEGI in the highly vascularized areas were identified by scanning the corneal sections using the 10 × objective. Individual immunostained vessels were counted using a microscope (E-400; Nikon, Tokyo) equipped with a DS-R1i video camera (DS-U3; Nikon) using the 40 × objective, 0.3502 mm²/field. Five fields in each section were evaluated by image analysis using the NIS Elements D Imaging Software (Microvision, Evry, France). The positive immunoreactions for VEGF and its receptors, and for VEGI that were observed in the vessels were graded blindly by two independent observers (S. Altan and H. Sagsoz).

Values obtained from five different areas of the corneal samples from each rabbit were summed and that value was recorded for each animal. Each slide was examined at least twice by the same observer at an interval of 2 weeks.

**Statistical analysis**

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, v. 15.0). The non-parametric Kruskal–Wallis test was used to

### Table 1. Details of antibodies used

<table>
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<th>Antibody</th>
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<th>Dilution</th>
<th>Supplier, lot number</th>
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<tr>
<td>Flt4</td>
<td>C-20, polyclonal IgG Rabbit 1:400 Santa Cruz Biotechnology, sc-321</td>
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<td></td>
<td></td>
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<tr>
<td>VEGI</td>
<td>FL-174, polyclonal IgG Rabbit 1:400 Santa Cruz Biotechnology, sc-32945</td>
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</tbody>
</table>

VEGF, vascular endothelial growth factor; Flk1/KDR, kinase insert domain-containing region; Flt1/fms, fms-like tyrosine kinase; Flt4, fms-related tyrosine kinase 4; VEGI, vascular endothelial growth inhibitor factor.
determine significant differences between the groups or different groups of cells. The Mann–Whitney U-test was used to determine in which particular groups of cells the intensity scores significantly differed from one another. Pearson’s analysis was used to investigate the correlations between the parameters. For all statistical evaluations, values for \( p \leq 0.05 \) were considered statistically significant.

**Results**

**Growth pattern of new blood vessels in the cornea**

CNV developed following induction of a corneal burn in 96% of cases. All of the blood vessels originated from the limbal region and extended toward the center of the cornea. In all groups on day 2 post-treatment, new arc-like or anisiform blood vessels, 0.2–0.4 mm long, had grown toward the cornea. Because the NV areas were in contact with the limbal cornea, however, identifying them at the microscopic level was difficult. By day 7, the blood vessels exhibited an increased amount of branching and the length of the new blood vessels had reached 0.6–0.8 mm. The NV areas were clearly larger in the indomethacin and DMSO + indomethacin treated groups, and exhibited dense ramification. On day 14, there were fewer NV areas in the control and DMSO-treated groups, but the length of the new blood vessels had grown to 1.5–2.5 mm in the indomethacin and DMSO + indomethacin treated groups. Branching of newly formed blood vessels had decreased, however, and these vessels terminated in dead ends (Fig. 1, Table 2).

**Microscopic development of NV during corneal inflammation**

Inflammation developed after induction of a corneal burn with HF in all treatment groups. The severity of inflammation was increased in the indomethacin and DMSO + indomethacin treated groups, and a large number of inflammatory cells had infiltrated into the corneal stroma on days 7 and 14 post-treatment. On day 2 post-treatment, NV areas had formed in the stromal area adjacent to the corneal limbus in all treatment groups. By day 7, the newly formed blood vessels had extended to the center of the cornea and were localized either immediately beneath the epithelium or deep in the stroma where the acid burn occurred. The number of the newly formed blood vessels was greater in the control, indomethacin and DMSO + indomethacin treated groups than in the DMSO treated group \((p < 0.05)\). On day 14, new blood vessels were observed in the stroma in the NV areas. In the control and DMSO treated groups, the number of blood vessels was less than in the indomethacin and DMSO + indomethacin treated groups \((p < 0.05)\) (Figs. 2–6, Table 2).

Pathological lymph vessels formed in the corneal stroma adjacent to the limbus in all groups after development of NV. On days 7 and 14 post-treatment, the density of the lymph vessels was increased in the superficial and deep stroma in the area of the acid burn (Figs. 2–6).

**Expression of VEGI and VEGF/VEGFR in the blood vessels of the NV areas**

VEGI, VEGF and VEGF receptors were expressed to varying degrees in the nucleus and cytoplasm of the endothelial cells in the blood vessels of the NV areas. In indomethacin and DMSO + indomethacin treated groups, VEGF and its receptors were expressed in the majority of the pathological vessels in the NV area on days 7 and 14 rather than on day 2; this difference in the level of expression on different days post-treatment was statistically significant \((p < 0.05)\) (Table 2). The expression of VEGF and its receptors was stronger in the blood vessels in the NV areas on days 7 and 14 in the control group and on day 7 in the DMSO treated group compared to the expression on other post-treatment days. The difference in the level of expression on the different post-treatment days, however, was not statistically significant (Figs. 2–5). The relative number of VEGI-positive blood vessels was similar in the control, DMSO- and DMSO + indomethacin treated groups throughout the post-treatment period. In the indomethacin treated group, however, the number of VEGI-positive vessels was greater on day 14 than on days 2 and 7 \((p < 0.05)\) (Fig. 6). Lymph vessels exhibited varying levels of flt4 expression in all groups; however, the greatest expression was observed in the indomethacin and DMSO + indomethacin treated groups (Fig. 5).

**Expression of VEGI and VEGF/VEGFR in the cornea**

Nuclear VEGF (Fig. 2), flk1/KDR (Fig. 3), flt1/fms (Fig. 4) and flt4 (Fig. 5) was present in some of the cells of the basal epithelium. The expression of VEGF (Fig. 2), flk1/KDR (Fig. 3) and flt1/fms (Fig. 4) was greater in the regions of epithelial repair. The epithelial and stromal cells of the control group on day 7 and the inflammatory cells of the indomethacin and DMSO + indomethacin treated groups on days 7 and
14 exhibited stronger expression of VEGF than the corneal endothelial cells; the difference was statistically significant ($p < 0.05$) (Fig. 2, Table 3). Furthermore, the expression of flk1/KDR and flt4 was stronger in the stromal cells of the control group on days 2 and 14 and in the epithelial and inflammatory cells of the indomethacin and DMSO + indomethacin treated groups on day 14 than that of the corneal endothelial cells ($p < 0.05$) (Figs. 3, 5, Table 3). Conversely, the expression of flt1/fms was stronger in the epithelial, stromal and inflammatory cells of the cornea on days 7 and 14 in the control group, the inflammatory cells of the indomethacin treated group on day 14, and the DMSO + indomethacin treated groups on day 7 compared to the endothelial cells ($p < 0.05$) (Fig. 4, Table 3). The epithelial, stromal and inflammatory cells of the cornea exhibited stronger VEGF and VEGF receptor expression than the corneal endothelial cells in the DMSO treated group; however, the difference was

![Digitally enhanced slit-lamp images of rabbit corneas after HF burn showing new vessels invading the cornea. Growth patterns of corneal neovascular vessels on a) day 2, b) day 7, and c) day 14 for the control; on a) day 2 of the application, b) day 7 of the application, and c) day 14 of the application for topical treatment with 0.1% indomethacin; on a) day 2 of the application, b) day 7 of the application, and c) day 14 of the application for topical treatment with 40% aqueous DMSO eye drops; on a) day 2 of the application, b) day 7 of the application, and c) on day 14 of the application for topical treatment with indomethacin + DMSO solution. The limbus and the direction of vessel growth are indicated (bars = 3 mm).](image-url)
not statistically significant (Figs. 2–5, Table 3). The expression of VEGI was stronger on days 7 and 14 in the stromal cells of the control group, on days 2 and 14 in the stromal cells of the DMSO treated group and on day 14 in the inflammatory cells of the DMSO + indomethacin treated group compared to VEGI expression in the corneal endothelial and epithelial cells; these differences in expression were statistically significant (p < 0.05) (Fig. 6, Table 3).

VEGI, VEGF and VEGF receptors were expressed moderately in the normal corneal epithelial, endothelial and stromal (fibrocyte/fibroblast) cells. Staining was absent in all negative controls (Fig. 7).

Correlation between expression of the ligands and receptors

The correlations between the ligands (VEGF and VEGI) and their receptors are shown in Table 4. Significant positive within-group correlations for the expression of the ligands and their receptors were found between the post-treatment days (Table 4). The correlation of the expression of VEGF and VEGI between the same groups was strongly positive (control group, r = 0.367, p < 0.01; indomethacin treated group r = 0.393, p < 0.01; DMSO treated group r = 0.240, p < 0.05; DMSO + indomethacin treated groups r = 0.317, p < 0.05).

Discussion

We found that in the rabbit, application of topical DMSO and angiogenic cytokines played important roles in corneal repair and healing of CNV following chemical burns of the cornea caused by HF.

The role of DMSO and indomethacin in corneal repair

Corneal epithelial and stromal repair after trauma such as chemical burns involves migration,
proliferation and differentiation of stem cells as described previously (Comptour et al. 2016).

DMSO alone can be used to treat conditions such as amyloidosis and chemical burns as an anti-inflammatory agent and for scavenging reactive oxygen species. Culturing pluripotent stem cells in aqueous DMSO increases the proportion of cells in the early G1 phase of the cell cycle and subsequently improves their competency for differentiation into multiple lineages (Chetty et al. 2013, Ogaki et al. 2015).

VEGF and its receptors are increased in epithelial and endothelial cells, and keratinocytes (stromal cells) of the cornea throughout the inflammatory process; inflammation may regulate the synthesis of VEGF and its receptors (Amano et al. 1998, Bock et al. 2007, Chang et al. 2001, Cursiefen et al. 2000, Gan et al. 2004, Totan et al. 2015).

Fig. 2. Immunohistochemical localization of VEGF in rabbit cornea after HF burn. Localization of epithelial, stromal, and inflammatory cells of cornea on a) day 2, b) day 7, and c) day 14 for the control; on a) day 2 of the application, b) day 7 of the application, and c) day 14 of the application for topical treatment with 0.1% indomethacin; on a) day 2 of the application, b) day 7 of the application, and c) day 14 of the application for topical treatment with 40% aqueous DMSO eye drops; on a) day 2 of the application, b) day 7 of the application, and c) day 14 of the application for topical treatment with indomethacin + DMSO solution. E, epithelium; S, stroma; V, blood vessels; L, lymphatic vessels; arrowheads, positive stromal and inflammatory cells (bars = 25 µm).
VEGF and its receptors perform many functions including regulating the functions of epithelial cells. In non-endothelial cells, such as epithelial cells and myocytes, VEGF and its receptors can act as anti-apoptotic, growth or pro-survival factors (Amano et al. 1998, Bock et al. 2007, Cursiefen et al. 2000, Lejbkowicz et al. 2005, Totan et al. 2001, Walsh and Grant 1997, Yan et al. 2007).

We found that VEGF and its receptors, flk1/KDR, flt1/fms and flt4, played a similar role in the cornea of the DMSO, indomethacin and DMSO + indomethacin treated groups (Table 3). We found significant positive correlations between the expression of flk1/KDR, flt1/fms, flt4 and VEGF in corneal epithelial, stromal and inflammatory cells ($p < 0.05$) (Table 4). Our findings support the hypothesis that VEGF, by way of
its receptors, regulates growth, cell survival and repair, and prevents apoptosis in the epithelial, endothelial and stromal cells of the rabbit cornea after treatment with DMSO. Angiogenic factors also are expressed by the cell nucleus. In humans, nuclear VEGF may play an important role in wound healing and tissue repair by inducing the coagulation and fibrinolysis pathways (Lejbkowicz et al. 2005). Our findings suggest that the expression of nuclear VEGF and its receptors by the epithelial, stromal and endothelial cells of the rabbit cornea serve a similar purpose.


Fig. 4. Immunohistochemical localization of flt1/fms in rabbit cornea after HF burn. Localization of epithelial, stromal, and inflammatory cells of cornea on a) day 2, b) day 7, and c) day 14 for the control; on a) day 2 of the application, b) day 7 of the application, and c) day 14 of the application for topical treatment with 0.1% indomethacin; on a) day 2 of the application, b) day 7 of the application, and c) day 14 of the application for the topical treatment with 40% aqueous DMSO eye drops; on a) day 2 of the application, b) day 7 of the application, and c) day 14 of the application for topical treatment with indomethacin + DMSO solution. E, epithelium; S, stroma; V, blood vessels; L, lymphatic vessels; arrowheads, positive stromal and inflammatory cells (bars = 25 µm).
We found that VEGF, flk1/KDR, flt1/fms and flt4 were expressed strongly in the inflammatory cells of all groups on days 2, 7 and 14 post-treatment. We also found that the level of expression of these factors in inflammatory cells compared to epithelial, stromal and Descemet membrane endothelial cells was higher on days 7 and 14 in the indomethacin and DMSO + indomethacin treated groups, but not in the DMSO treated group \((p < 0.05)\) (Table 3). Macrophages in the stroma of inflamed and neovascularized corneas strongly express angiogenic cytokines. Therefore, macrophages may play an important role in the pathogenesis of CNV by either directly secreting VEGF/VEGFR or inducing the secretion of VEGF with the assistance of cells such as epithelial cells and keratinocytes (Totan et al. 2001).

Based on our findings, we suggest that angiogenic cytokines respond to other inflammatory mediators, such as interleukin-1, 8, leukotriene,
matrix metalloproteinase 9, and support CNV by increased synthesis of VEGF and its receptors in inflammatory cells. Also, DMSO exerts an anti-inflammatory effect by inhibiting the influx of polymorphonuclear cells and monocytes into the sites of inflammation and by stimulating prostaglandin synthesis. DMSO inhibits oxygen free radicals, which increases the migration of inflammatory cells to the site of inflammation and therefore decreases the production of oxygen free radicals (Altan and Ogurtan 2016). The lower expression of VEGF and its receptors in the inflammatory cells of the DMSO treated group that we found indicates that the anti-inflammatory effect of DMSO was robust after topical application.

The expression of VEGI was stronger in the stromal and inflammatory cells than in the endothelial and epithelial cells in all groups with a neovascularized cornea ($p < 0.05$) (Table 3). The lack of information on the expression of VEGI in
<table>
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<td>Control</td>
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<td>Day 14</td>
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<td>Dimethyl sulfoxide + Indomethacin</td>
<td></td>
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</tr>
<tr>
<td>Day 2</td>
<td>3.50 ± 0.55</td>
<td>3.0 ± 0.0</td>
<td>4.0 ± 0.0</td>
<td>3.0 ± 0.0</td>
</tr>
<tr>
<td>Day 7</td>
<td>3.0 ± 0.0</td>
<td>3.0 ± 0.0</td>
<td>4.0 ± 0.0</td>
<td>3.0 ± 0.0</td>
</tr>
<tr>
<td>Day 14</td>
<td>2.75 ± 0.50</td>
<td>2.50 ± 0.50</td>
<td>2.0 ± 0.0</td>
<td>2.0 ± 0.0</td>
</tr>
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</table>

Different superscripts a, b, c, d in the same line indicate significant differences (p < 0.05). VEGF, vascular endothelial growth factor; Flk1/KDR, the kinase insert domain-containing region; Flt1/fms, the fms-like tyrosine kinase; Flt4, fms-related tyrosine kinase 4; VEGI, vascular endothelial growth inhibitor factor; EC, epithelial cells; SC, stromal cell; DME, descemet membrane endothelial cell; IC, inflammatory cells.
rabbits makes it difficult to delineate the exact role of VEGI in the non-endothelial cells of the normal and NV cornea. Based on the available literature, however, we propose that in the non-endothelial cells of the rabbit cornea, VEGI may act as a signaling protein that maintains the balance between cell proliferation and apoptosis (Wang and Wang 2010). Based on the similarity of expression of VEGF and its receptors to that of VEGI in the epithelial, inflammatory and stromal cells of the NV cornea, and the presence of positive significant correlations between VEGI and VEGF (Table 4), we suggest that the function of VEGI in the NV rabbit cornea is to regulate the expression of VEGF and

![Fig. 7. Localization of VEGF, flk1/KDR, flt1/fms, flt4, and VEGI in healthy corneal epithelial and stromal fibrocyte/fibroblast cells in the control, topical treatment with 0.1% indomethacin, topical treatment with 40% aqueous DMSO eye drops, and topical treatment with indomethacin + DMSO solution. No immunostaining of VEGF, flk1/KDR, flt1/fms, flt4, and VEGI is seen in the rabbit cornea. E, epithelium; S, stroma (bars = 25 µm).](image)

![Table 4. Correlations between VEGF as well as, and VEGI and Flk1/KDR, Flt1/fms, Flt4 receptors in the rabbit cornea with HF acid burn](image)
its receptors. Metheny-Barlow and Li (2006) reported that VEGI also can regulate the expression of several important genes involved in angiogenesis.

The role of angiogenic factors in CNV


Consistent with earlier reports using alkali induced burns in animal models, our findings indicate that the NV areas emerged during the early phase of inflammation and reached a peak by days 7 and 14 in the indomethacin and DMSO + indomethacin treated groups. We also found that corneal inflammation caused by the HF burn was severe. Shi et al. (2011) reported that the size of the CNV area is correlated positively with the severity of inflammation and the density of inflammatory cells. Therefore, we infer that the differences in the size of the CNV areas among the groups may be related to the severity of inflammation. We found that the number of inflammatory cells and the size of the NV areas were decreased in the DMSO treated group compared to those in the other groups, which indicates that administration of DMSO inhibited the development of inflammation, which consistent with the report by Shi et al. (2011).

The VEGF/VEGFR pathway is important for induction of neovascularization and lymphangiogenesis. VEGF, particularly VEGFA, CNV (Amano et al. 1998, Burgher et al. 2011, Chang et al. 2001, Cursiefen et al. 2000, Kim et al. 2010, Onder et al. 2014, Ozdemir et al. 2014), VEGFC, VEGFD and VEGFR3 (flt4) family was expressed at varying levels in the blood vessels located in the NV areas. The expression of VEGF and its receptors was increased in many vessels located in the NV area in the indomethacin and DMSO + indomethacin treated groups on days 7 and 14, and this level of expression was statistically significant (p < 0.05) (Table 2). VEGF and its receptors were expressed in the vessels located in the CNV areas, which indicates that these factors stimulated and maintained the growth of new blood and lymph vessels in the cornea, which is consistent with previous reports (Amano et al. 1998, Burgher et al. 2011, Chang et al. 2001, Cursiefen et al. 2000, Kim et al. 2010, Onder et al. 2014, Ozdemir et al. 2014, Totan et al. 2001, Yan et al. 2007, Zhang and Zhang 2008). The nuclear expression of VEGF and its receptors was found in the endothelial cells of the vessels in all groups. We suggest that VEGF and its receptors increased the growth and permeability of endothelial cells as reported by Lejbkowicz et al. (2005).

We determined that following HF induction of a corneal burn, new lymph vessels grew from the limbus toward the center of the corneal burn in all groups. On days 7 and 14, we observed that flt4 exhibited intense expression in the endothelial cells, particularly in the newly formed vessels. The correlation between newly formed blood and lymph vessels in the cornea, however, remains controversial. Cursiefen et al. (2000) and Shi et al. (2011) observed that in mice, corneal lymphangiogenesis developed following development of CNV. Cursiefen et al. (2000) speculated that VEGFC produced by the endothelial cells of the newly formed vessels induced the branching of lymph vessels from the limbus toward the center of the cornea, which led to corneal lymphangiogenesis. Consistent with the reports by Cursiefen et al. (2000) and Shi et al. (2011), we found that the formation of new blood vessels was followed by growth of new lymph vessels and this was confirmed by flt4 immunoreactivity.

VEGI inhibits endothelial cell proliferation and angiogenesis (Zhang et al. 2010). Corneal NV develops either because of the deficiency of factors such as angiotatin, endostatin, and pigment epithelium-derived factor or because of inhibition of the activities of these factors (Bock et al. 2007, Chang et al. 2001, Kim et al. 2010, Onder et al. 2014, Zhang et al. 2005). We found that in CNV in rabbits, VEGI immunoreactivity appeared in the endothelial cells of the blood vessels, including

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the venous and arterial vessels, in all groups. In the indomethacin treated group, the number of VEGI-positive vessels in the NV areas was greater on day 14 post-treatment than on days 2 and 7 post-treatment \( (p < 0.05) \). Earlier reports suggest that VEGI, which is secreted by endothelial cells, may function as an autocrine inhibitor of angiogenesis (Duan et al. 2012, Zhang et al. 2010). Our findings also indicate that VEGI may have an anti-angiogenic role in the blood vessels of the CNV in rabbits. Further investigations are required to test this hypothesis concerning the role of VEGI in the rabbit cornea.

**Role of topical drug administration in the inhibition of CNV**

Several medical and surgical treatment methods have been described for controlling CNV. The conventional method for suppressing actively developing NV is administration of topical steroids. Anti-VEGF agents and non-steroidal agents such as rapamycin, bevacizumab, ranibizumab, and pegaptanib have been used clinically. Anti-VEGF agents, alone or in combination with steroids, have been used to treat CNV (Bock et al. 2007, Kim et al. 2010, Onder et al. 2014, Ozdemir et al. 2014, Totan et al. 2001, Yan et al. 2007). Despite numerous treatment modalities, however, there is no consensus regarding the best treatment (Balicki 2012). We administered the non-steroidal anti-inflammatory drug, indomethacin, and the antioxidant, anti-inflammatory agent, DMSO, separately and in combination to investigate whether these agents inhibited CNV. Because DMSO exhibits low toxicity and can penetrate biological membranes readily, it is the preferred treatment for ocular diseases. DMSO also exhibits analgesic, weak bacteriostatic, anticoagulant, anti-inflammatory, and free radical scavenging activities (Balicki 2012). Indomethacin also is used for treating ocular diseases. Indomethacin shows an effect similar to or stronger than that of steroids, particularly when used for the treatment of acute alkaline corneal burns (Laria et al. 1997).

NV occurs first in all pathological lesions of the cornea. Therefore, a decrease in CNV is a helpful prognostic indicator for the regression of ocular diseases. We found significant differences in the numbers of the vessels between the control and the treatment groups in the NV areas of the cornea. Compared to the control group, we found that the number of blood vessels in the DMSO group were decreased, but were increased in indomethacin- and indomethacin + DMSO–treated groups. In previous studies, ophthalmic DMSO drops were used at concentrations of 20%, 40%, 50%, or 60% or in combined forms, for example, 0.2% fluconazole, 1% itraconazole, and then suppression of CNV was observed (Altan and Ogurtan 2016, Balicki et al. 2008, Balicki 2012).

We found that administration of DMSO alone was more effective for inhibiting CNV than administration of indomethacin alone or the combination of indomethacin and DMSO. On days 7 and 14, after topical administration of 40% (v/v) aqueous DMSO, marked inhibition of the blood vessels was observed, which can be attributed to the anti-inflammatory effect of DMSO. Our findings in the group treated with DMSO only were similar to those in previous studies in which corticosteroids and cyclo-oxygenase inhibitors were administered to inhibit CNV (Kim et al. 2010); our findings suggest that DMSO can be used as an alternative to these agents. Indomethacin commonly is used to suppress intra-operative myosis, cystoid macular edema, and various inflammations. In addition, indomethacin is used as an alternative to steroids for treating alkaline burns of the eye owing to its anti-inflammatory and antioxidant effects (Altan and Ogurtan 2016). For corneal alkaline burns, 1% (w/v) indomethacin combined with dexamethasone produced positive results (Laria et al. 1997). Unlike alkaline corneal burns (Laria et al. 1997), administration of indomethacin alone or combined with DMSO was ineffective for inhibiting HF-induced CNV in rabbits. These differences may be due to differences in indomethacin concentration and dose. Laria et al. (1997) used 1% (w/v) indomethacin, while we used a commercially available 0.1% (w/v) indomethacin solution, because the 1% solution was unavailable. Moreover, because indomethacin is derived from indoleacetic acid, it is used as the sodium and trometamine salts for ophthalmology. Indomethacin is not stable in an alkaline medium and it is weakly soluble in an acidic medium. We suggest that the ineffectiveness of indomethacin alone or in combination with DMSO may be related to these factors.

VEGF and its receptors are expressed in the endothelial, epithelial, and stromal cells of the cornea. Our findings suggest that these angiogenic factors, together with the application of DMSO, can be effective for stimulating repair of corneal damage. Furthermore, we determined that angiogenic cytokines stimulated CNV by enabling the growth of new blood and lymph vessels in the inflamed cornea. The expression of VEGI in the damaged cornea suggests that VEGI can maintain a balance between cell proliferation and apoptosis, and inhibit CNV. Furthermore, we found that topical
administration of DMSO alone was more effective than indomethacin for inhibiting CNV.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References


