• Basic Research •

Effect of sorafenib in a murine high risk penetrating keratoplasty model

Yang Kyung Cho[1], Eun Young Shin[2], Hironori Uehara[3], Balamurali K Ambati[3]

[1]Department of Ophthalmology, St.Vincent's Hospital, College of Medicine, the Catholic University of Korea, Suwon, Gyeonggi-Do 16247, Korea
[2]Research Institute of Medical Science, St.Vincent's Hospital, College of Medicine, the Catholic University of Korea, Suwon, Gyeonggi-Do 16247, Korea
[3]Department of Ophthalmology, University of Utah, School of Medicine, Salt Lake City, Utah 84132, USA
Correspondence to: Yang Kyung Cho. Department of Ophthalmology, St.Vincent's Hospital, College of Medicine, the Catholic University of Korea, 93 Ji-Dong, Paldal-Gu, Suwon, Gyeonggi-Do 16247, Korea. yangkyeung@hanmail.net
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Abstract

• AIM: To evaluate the effect of sorafenib in murine high risk keratoplasty model.

• METHODS: Graft survival, corneal neovascularization, and corneal lymphangiogenesis were compared among the sorafenib, dexamethasone, dimethyl sulfoxide (DMSO), and phosphate buffered saline (PBS) groups following subconjunctival injection in mice that underwent high risk penetrating keratoplasty (HRPK). Real-time polymerase chain reaction was performed to quantify the expression of inflammatory cytokines and vascular endothelial growth factor (VEGF)-A, VEGF-C, vascular endothelial growth factor receptor (VEGFR)-2, VEGFR-3.

• RESULTS: The two-month graft survival rate for HRPK was 42.86% in sorafenib group, 37.50% in dexamethasone group, 0 in DMSO group, and 0 in PBS group. Sorafenib significantly increased graft survival compared to the DMSO and PBS group (*P*<0.05). The sorafenib didn't show significant effect in decreasing neovascularization compared with dexamethsone, DMSO, and PBS group. The sorafenib showed less total lymphangiogenesis than the dexamethasone, DMSO, and PBS group (*P*=0.011, *P*<0.001, *P*<0.001, respectively). The sorafenib group showed reduced expression of VEGF-C, tumor necrosis factor (TNF)-alpha, interleukin (IL)-6, VEGFR-2 and VEGFR-3 compared with DMSO group and PBS group (all *P*<0.05). The sorafenib group didn't show difference in the expression of VEGF-A compared with DMSO, neither with PBS. The sorafenib group showed reduced expression of VEGFR-3 compared with dexamethasone (*P*=0.051).

• CONCLUSION: The subconjunctivally administered sorafenib shows significant anti-lymphangiogenic effect, resulting in increased transplant survival in a murine high risk keratoplasty model. We suggest that a close linkage between decreased VEGF-C/VEGFR-2 and -3 signaling and increased corneal graft survival by sorafenib seems to exist.

• KEYWORDS: sorafenib; neovascularization; graft survival; lymphangiogenesis; dexamethasone

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INTRODUCTION

In penetrating keratoplasty (PK) in high risk patients, even with current immunosuppression, rejection rates can be over 70%, whereas PK in normal risk patients maintains the survival rates as high as 90% at the first year[1-4]. Compared to normal risk penetrating keratoplasty (NRPK), eyes with high risk penetrating keratoplasty (HRPK) exhibited much higher levels of (lymph) angiogenesis[2-4] and inflammatory chemokines in early postoperative period[3,5]. Risk for graft rejection can be corneal (lymph) angiogenesis, regrafts, high intraocular pressure, trauma, and perioperative inflammation. Corneal (lymph) angiogenesis is a well known risk for graft rejection and failure[3,5]. Therefore, minimizing corneal (lymph) angiogenesis has the potential to decrease immunologic graft rejection and graft failure rates[6-7]. Several anti-(lymph) angiognenic treatment was tried to enhance graft survival[7-8]. However, because lymphatics might play important roles to heal conjunctivitis or conjunctivalchemosis or probable corneal edema, anti-lymphangiogenesis (LY) treatment can affect the wound healing of these structures[9-10]. For example, rapamycin which can inhibit LY through inhibition of vascular endothelial growth factor (VEGF)-C can inhibit wound healing, even though its therapeutic effect for treatment of malignancy[10]. Especially in corneal transplantation, LY, not hemangiogenesis has been reported to be a primary mediator of rejection[7-8]. So, treatment targeting LY have been studied and developed

to treat different tumors and ocular diseases. However, the other report showed that anti-angiogenic treatment such as strong VEGF-A trap was more successful in improving long-term graft survival as compared with anti-lymphangiogenic treatment such as anti VEGF-C and soluble vascular endothelial growth factor receptor (VEGFR) 3[11]. The strong angiogenic VEGF-A increase hemangiogenesis through VEGFR-2 binding. The strong lymphangiogenic VEGF-C and -D are the main prolymphangiogenic factors that act through the activation of VEGFR-3[8,12]. When VEGF binds to VEGFR (receptor), then activation of the rat sarcom (RAS)/rapidly accelerated fibrosacrcoma (RAF)/extracellular signal regulated kinase (ERK)/mitogen activated protein kinase (MAPK) starts signal transduction which leads to endothelial proliferation[8,12]. Sorafenib is a potent inhibitor of RAS/RAF kinase and tyrosine kinases such as VEGFR-2, PDGFR β, and VEGFR-3[12-14]. This multikinase inhibitors interfere with the activation of VEGFRs by preventing phosphorylation. Sorafenib is already in use as an anticancer drug that aims at tumor proliferation and neovascularization (NV)[12-14]. Recent reports have suggested new therapeutic role of sorafenib in ocular disease; age-related macular degeneration (AMD) and retinopathy of prematurity (ROP)[15-16]. The effect of oral administration of sorafenib on choroidal and corneal NV was previously reported[17]. From the several previous reports of the effect of sorafenib in various type of tumor and neovascular disease in retina, we expected that sorafenib would work on cornea. We tried to evaluate the effect of sorafenib in neovascular disease of cornea, the HRPK, which has high graft failure rate due to severe hem/LY. Here, we studied the effect of subconjunctivally injected sorafenib on the graft survival, LY and hemangiogenesis in a mouse model of high risk corneal PK.

SUBJETCS AND METHODS

The experiments were performed with the regulations of Association for Research in Vision and Ophthalmology and approval by the Institutional Animal Care and Use Committee of the Catholic University of Korea, St. Vincent's Hospital.

High Risk Corneal Transplantation Recipient mice [6 to 8 weeks old, female, Bagg Albino (BALB)/c] and donor mice (C57BL/6) (the Koatech, Pyeongtak, Korea) were anesthetized by Zoletil®50 (30 mg/kg, Virbac Korea Co. Ltd.) and xylazine (10 mg/kg) was done. Prior to PK, two corneal sutures (10-0 nylon, CS140-6, Ethicon, Inc.) were placed between the corneal center and the limbus to induce vascularization. The corneal sutures were removed and corneal transplantation was done according to the methods used in the normal risk PK[18].

Subconjunctival Injection of Anti-angiogenic Agents Sorafenib (Santa Cruz Biotechnolology, Inc., Santa Cruz, CA, USA) was dialted with vehicle dimethyl sulfoxide (DMSO). In four group, the respective treatment; sorafenib (15 μL, 465 μg/mL, 12 eyes), dexamethasone (15 μL, 500 μg/mL, 12 eyes), DMSO (15 μL, 12 eyes) and phosphate buffered saline (PBS) (15 μL, 12 eyes) were injected into the subconjunctival space from the day of transplantation and weekly to postoperative 8wk. Among these groups, sorafenib group was the case group. And the dexamethasone, which is already known as antiangiogenic agent was the positive control group. The sorafenib was diluted with vehicle DMSO, so the DMSO was the negative control. PBS group was another negative control group.

Clinical Evaluation of Rejection Microscopic examination was done weekly through post-op week 8 and corneal microscopic pictures were taken. Evaluation of graft clarity according to the grading system was done as previously described. The opacity grading (0 to 5) was as a previous report[19]: grades 3 and above were considered a graft rejection.

Analysis of Angiogenesis and Lymphangiogenesis To know the extent of corneal NV and LY in the recipient cornea before grafting, two corneal sutures were placed on 6 corneas between the corneal center and the limbus. After two weeks, we harvested 6 corneas to evaluate the extent of corneal NV before grafting. After immune staing with CD31 and LYVE-1, we evaluated the extent of NV and LY under fluorescent microscope. After PK and the planned injections for observation periods (8wk after PK), we harvested eyes and the corneas were trimmed. Vascular and lymphatic endothelial cells were immunestained on corneal flat mounts as our previous report[20]. After immune staining and flat mounting of the cornea, images of the corneal vasculature were captured by a fluorescent microscope (OLYMPUS BX51, Tokyo, Japan). NV and LY were quantified as a previous report[20]. Total NV (%)=neovascularized area/total cornea area×100%; total LY (%)=LY area/total cornea area×100%.

Comparison of Graft Survival, Angiogenesis and Lymphangiogenesis in High Risk Penetrating Keratoplasty The four groups (sorafenib, dexamethasone, DMSO and PBS) were compared in HRPK. Graft survival, NV and LY were compared.

Quantitative Real-time Polymerase Chain Reaction Analysis of Gene Expression in the Mouse Cornea After harvesting, the corneas were trimmed and the expression of VEGF-A, VEGF-C, VEGFR-2, VEGFR-3, tumor necrosis factor (TNF)-alpha and interleukin (IL)-6 was analyzed using real-time polymerase chain reaction (RT-PCR) as previous report[20]. We used published primer sequences for mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH)[20], VEGF-A[21], VEGF-C[21], VEGFR2[21], VEGFR3[21], TNF-alpha[22], and IL-6[23]. Each gene expression level was analyzed by the Ct method, using GAPDH expression as an internal control. The relative expression level of each sample is expressed as a fold change compared to the normal control (PBS).

Statistical Analysis SPSS 11.5 (Chicago, IL, USA) was used. Graft survival was analyzed using Kaplan-Meier survival

curves (log rank test). NV and LY was compared with groups using an unpaired two-tailed *t*-test. RT-PCR results were compared using ANOVA (analysis of variance) with post hoc test and the unpaired *t*-test. A $P<0.05$ was considered statistically significant.

RESULTS

Graft Survival Figure 1 shows the comparison of graft survival among the four groups in HRPK. There was no difference in graft survival between the sorafenib and the dexamethasone groups ($P>0.05$). Sorafenib significantly increased graft survival compared to the DMSO and PBS ($P=0.023$, $P=0.022$, respectively). Dexamethasone showed increased graft survival compared to the DMSO and PBS ($P=0.082$, $P=0.115$, respectively), but they don't reach statistical significance. The graft survival was not different between the DMSO and PBS ($P>0.05$). At the postoperative eight-week, the graft survival rate for each group was 42.86% in sorafenib, 37.50% in dexamethasone, 0 in DMSO, and 0 in PBS. The subconjunctivally administered sorafenib showed increased transplant survival in a murine high risk keratoplasty model.

Neovascularization Two weeks after corneal suture, before grafting, the the hemangiogenesis area in the recipient was 7.38% (mean) of total corneal area. Eight weeks after PK, the sorafenib group (13.33%±4.03%) didn't decreased NV significantly than DMSO group (18.64%±1.74%) ($P=0.232$). Dexamethasone group (13.91%±1.68%) showed less total neovascularized area than DMSO group (18.64%±1.74%), but they don't reach statistical significance ($P=0.087$) (Figures 2 and 3). Sorafenib and dexamethasone were no different with regard to their effects on NV. Similarly, there was no difference between DMSO and PBS (18.17%±1.31%) with regard to NV. Sorafenib showed negligible anti-angiogenesis effect compared with dexamethasone, DMSO and PBS LY. Two weeks after corneal suture, before grafting, the LY area in the recipient was 5.16% (mean) of total corneal area. Eight weeks after PK, The sorafenib group (5.79%±0.81%) showed less total lymphangiogenenic area than dexamethasone (12.30%±1.88%), DMSO (18.26%±1.39%), and PBS (18.55%±1.23%) group ($P=0.011$, $P<0.001$, $P<0.001$, respectively) (Figures 2, 3). The dexamethasone group showed less LY compared to the DMSO and PBS group ($P=0.035$, 0.043, respectively). There was no difference of LY between DMSO and PBS. Sorafenib has significant anti-LY effect on cornea compared with dexamethasone, DMSO and PBS.

Real-time Polymerase Chain Reaction The mRNA expression of VEGF-A, VEGF-C, TNF-alpha, IL-6, VEGFR-2 and VEGFR-3 in each group are shown in Figure 4. The mRNA expression ratios of VEGF-A, VEGF-C, TNF-alpha, IL-6, VEGFR-2 and VEGFR-3 are expressed normalized to

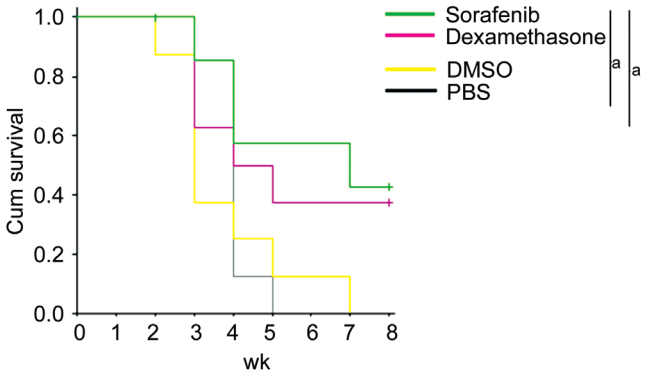

Figure 1 Comparison of graft survival in four groups: sorafenib, dexamethasone, DMSO, and PBS [a]$P<0.05$.

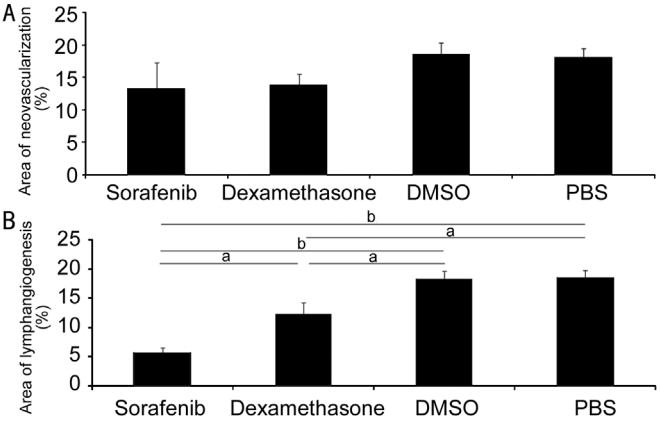

Figure 2 Comparison of NV and LY in four groups: sorafenib, dexamethasone, DMSO, and PBS A: Comparison of NV; B: Comparison of LY. [a]$P<0.05$, [b]$P<0.01$.

GAPDH (PBS group=1.0). There was no significant difference of expression ratio of VEGF-A, VEGF-C, TNF-alpha, IL-6, VEGFR-2 and VEGFR-3 between DMSO and PBS group.

The sorafenib showed reduced VEGF-C, TNF-alpha, IL-6, VEGFR-2 and VEGFR-3 compared with DMSO group ($P=0.03$, 0.005, 0.006, 0.003, 0.003, respectively). The sorafenib showed reduced VEGF-C, TNF-alpha, IL-6, VEGFR-2 and VEGFR-3 compared with PBS ($P=0.004$, $P=0.001$, $P=0.002$, $P=0.005$, $P<0.001$, respectively). The sorafenib didn't show difference in the expression of VEGF-A compared with DMSO, neither with PBS. The sorafenib group showed reduced expression of VEGFR-3 compared with dexamethasone ($P=0.051$) which is already well known anti-(lymph) angiogenic and anti-inflammatory agent. The dexamethasone group showed reduced VEGF-A, TNF-alpha, VEGFR-2 compared with DMSO group ($P=0.004$, $P<0.001$, $P=0.012$, respectively). The dexamethasone group showed reduced VEGF-A, VEGF-C, TNF-alpha, IL-6, and VEGFR-2 compared with PBS group ($P=0.007$, $P=0.040$, $P<0.001$, $P=0.016$, $P=0.017$, respectively). The dexamethasone group didn't show difference in the expression of VEGFR-3 compared with DMSO, neither with PBS.

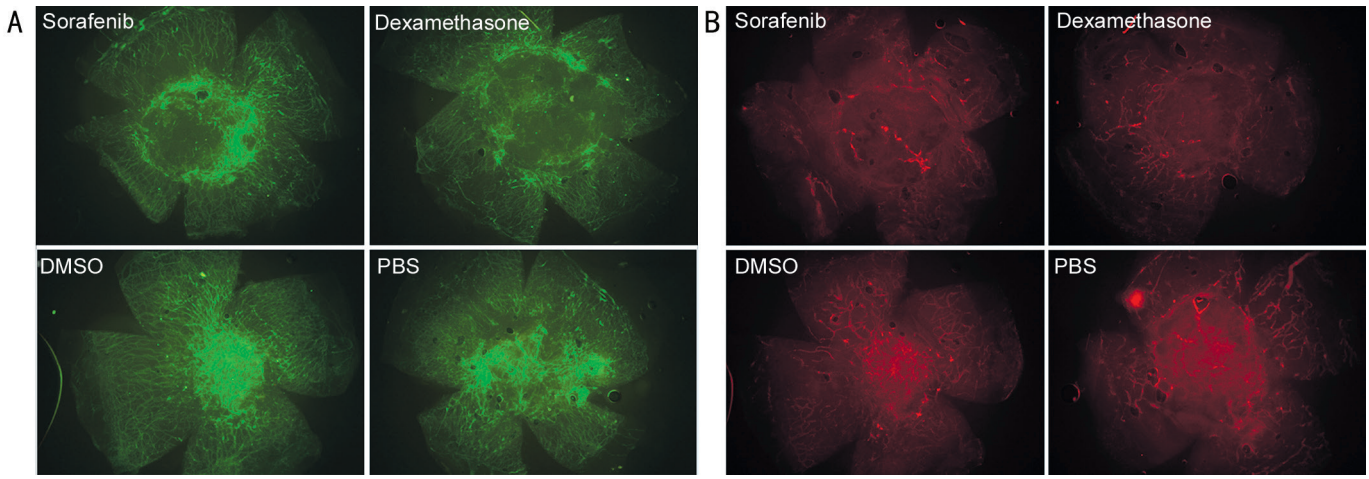

Figure 3 Representative pictures of NV and LY in four groups: sorafenib, dexamethasone, DMSO, and PBS A: CD31 staining, staining of blood vessel; B: LYVE-1 staining, staining of lymphatic vessel.

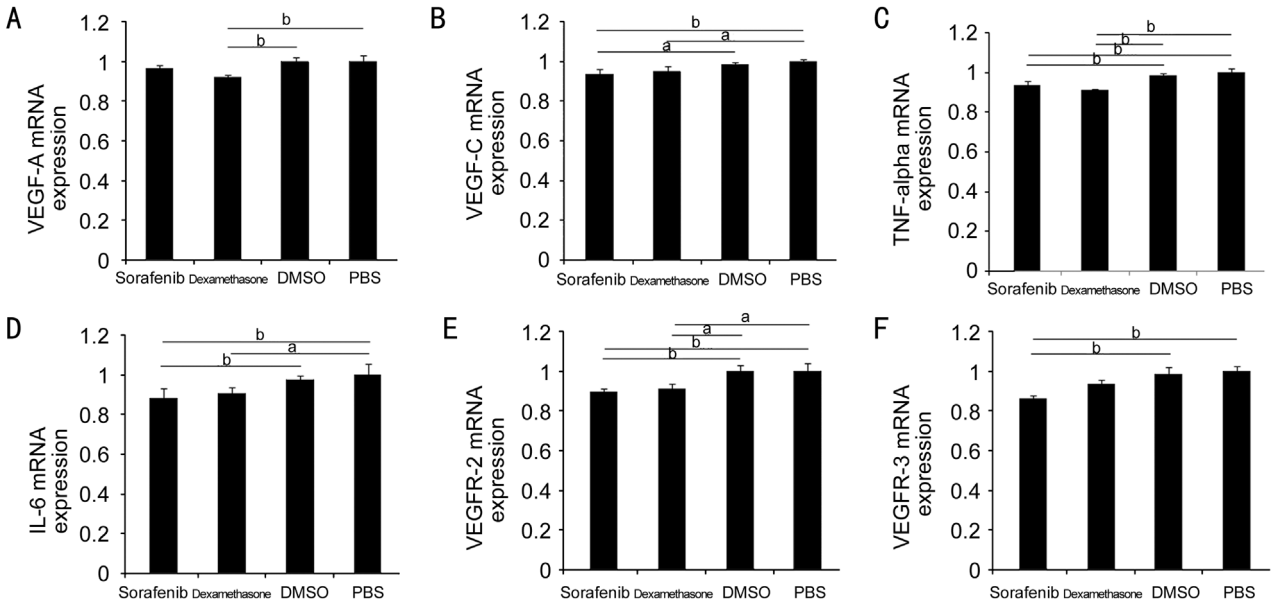

Figure 4 Comparison of mRNA expression in four groups: sorafenib, dexamethasone, DMSO, and PBS A: VEGF-A; B: VEGF-C; C: TNF-alpha; D: IL-6; E: VEGFR-2; F: VEGFR-3. $^{a}P<0.05$, $^{b}P<0.01$.

DISCUSSION

Among human transplantation surgeries, corneal transplantation is one of the most commonly performed. The overall 10-year survival rates of corneal grafts reach between 75% and 80%[1-3,5]. However, in the "high-risk" conditions, the survival rate drops to 30%-50% at 3 to 5-year follow-up[2,11,24]. Compared to normal risk keratoplasty, eyes with HRPK exhibited significantly higher levels of NV, LY[25] and inflammation in the early postoperative period[3,5,25-26]. The chemokines expressed in high risk eyes correlated with increased number of inflammatory cells in high risk recipients[26].

In this study, IL-6 and TNF-alpha, the inflammatory cytokines were significantly decreased by sorafenib as effectively as dexamethasone, even in HRPK which is different from normal risk PK in respect to their postoperative NV and inflammation. The most common possible reason for graft failure is immunologic rejection. The conditions that may place the cornea at a higher risk of rejection are corneal NV, LY, position of the graft close to limbus, and herpes simplex keratitis[5,27]. It has been reported that the preexisting blood and lymphatic vessels in cornea is a strong risk factor for immune rejection[28-29].

Ocular immune privilege can be acquired through avascularity, alymphatics, low major histocompatibility complex and native immunosuppression[3,5]. Although the normal cornea does not have blood and lymphatic vessels, NV and LY can be induced after traumatic, chemical, inflammatory or infectious damage. LY especially constitutes the afferent arm of the corneal transplantation immunity, and recently, it has been demonstrated that LY is a primary mediator of corneal transplant rejection[8,30]. So, decreasing lymphangiognesis can enhance graft survival. The VEGF is the a complex network controlling blood and lymphatic vessels[31-33]. Several previous

studies have shown that VEGFR-3 mediates LY in the cornea and other tissues[34-35]. Most recently, it has been indicated that VEGFR-2 also plays a role in corneal LY, but with unknown mechanism[30,34-35].

Our study evaluated the anti-angiognenic and anti-lymphangiogenic effect of sorafenib, as a therapeutic option against graft rejection in high risk keratoplasty. Sorafenib, a multikinase inhibitor, has shown promising results for the treatment of advanced hepatocellular carcinoma in clinical trials. The mechanisms of sorafenib's antitumor activities have been well presented. Evidence has shown that sorafenib inhibits the rapidly accelerated RAF/ MAPK/ERK kinase (MEK)/ERK signal pathways and receptor tyrosine kinases, including VEGFR-2, VEGFR-3, Flt-3, c-KIT, and platelet-derived growth factor receptor (PDGFR). the blocking of VEGFR and PDGFR may account for the antiangiogenesis effect of sorafenib. Sorafenib contains hydrophilic amide groups, and lipophilic pyridine, and has good biological activity[12-13,36]. Sorafenib is characterized by good absorbability because of its small molecular weight and the strong tissue. Its long half-life could reduce intraocular injection times. Additionally, sorafenib is a synthetic urea derivative and the immunogenicity is low[12-13,36].

Recent reports have also suggested the role of sorafenib in the treatment of AMD and ROP[15-17]. Our study focused on the effect of sorafenib on graft survival after corneal transplantation. In comparison of RNA expression, the results of our study show clearly that sorafenib significantly reduced the VEGFR-2 and VEGFR-3 in murine corneas. This is of importance, because VEGFR-2 and 3 in particular plays an important role in the development of lymphatic vessels, and a close linkage between VEGF-C/VEGFR-2 and -3 signaling and corneal graft rejection seems to exist. The sorafenib group showed reduced VEGFR-3 compared with dexamethasone (P=0.051). We suggest that this result can explain the reduced LY in sorafenib group compared with dexamethasone. Sorafenib did not affect VEGF-A compared with DMSO and PBS (P=0.232, 0.087, respectively), resulting in negligible effect on corneal angiogenesis. In our experimental setup, dexamethasone did not affect VEGFR-3 (Figure 4F). Dexamethasone mainly affected VEGF-A and VEGFR-2.

Dual blockade of VEGFR-2 and another key lymphatic receptor, such as VEGFR-3 by sorafenib, will maximize the anti-lymphangiogenic effect in high risk corneas. In comparison of NV and LY, both sorafenib and dexamethasone showed more pronounced effect in decreasing LY rather than NV in our HRPK model. Moreover, sorafenib decreased LY than dexamethasone. This can explain the results of the enhanced graft survival compared to DMSO only in sorafenib group, not in dexamethasone group. Because LY is a key mediator of corneal transplant rejection, in the high-risk eyes, the rejection rate can be as high as 90%[7-9,30,37]. Unfortunately, many patients who need corneal transplantation fall into this high risk category, and there is little effective treatment for them even with steroid treatment.

Our study indicates that sorafenib may be able to replace the effect of steroid in the high-risk grafting beds, to improve the survival rate of high-risk transplants. The anti-lymphangiogenic effect of sorafenib was significantly higher than that of dexamethasone in HRPK set up in our study (P=0.011), which leads to increased graft survival. Also, our study might support the importance of LY on graft survival rather than hemangiogenesis, which warrants further investigation.

In conclusion, we investigated the significant anti-lymphangiogenic effect of subconjunctivally administered sorafenib (off-label use), a multi-target-receptor tyrosine kinase inhibitor, on increasing transplant survival in a murine high risk keratoplasty model. These results mandate further clinical investigation of sorafenib for corneal graft.

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Cho YK designed the study, performed the animal work and the experiment, wrote the manuscript. Shin EY assisted the animal work and the experiment. Uehara Hironori designed and revised the study. Ambati BK designed and revised the study.

Conflicts of Interest: Cho YK, None; **Shin EY,** None; **Uehara H**, None; **Ambati BK**, None.

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